Express Mail No. EV 417055392 US

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 51/00, 51/04, C07F 5/00

A1

(11) International Publication Number: WO 96/36367

(43) International Publication Date: 21 November 1996 (21.11.96)

(21) International Application Number: PCT/US96/07002

(22) International Filing Date: 16 May 1996 (16.05.96)

(30) Priority Data:
08/442,174 16 May 1995 (16.05.95) US

(71) Applicant: PURDUE RESEARCH FOUNDATION [US/US]; Room 328, 1650 Engineering Administration Building, West Lafayette, IN 47907-1650 (US).

(72) Inventors: LOW, Philip, Stewart; 5850 Farm Ridge Road, West Lafayette, IN 47906 (US). GREEN, Mark, A.; 495 Littleton Street, West Lafayette, IN 47906 (US). MATHIAS, Carla, J.; 495 Littleton Street, West Lafayette, IN 47906 (US). LEE, Robert, J.; 140-5 Airport Road, West Lafayette, IN 47906 (US). LEAMON, Christopher, P.; 5112 Wineberry Drive, Durham, NC 27713 (US). HORN, Mark, Alan; The Weksman Institute, Rutgers University, Piscataway, NJ 08855 (US). HEINSTEIN, Peter, Frederick; 1013 East 800 North, West Lafayette, IN 47906 (US).

(74) Agents: LAMMERT, Steven, R. et al.; Barnes & Thornburg, 1313 Merchants Bank Building, 11 South Meridian Street, Indianapolis, IN 46204 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: COMPOSITION AND METHOD FOR TUMOR IMAGING

(57) Abstract

A method is provided for enhancing transmembrane transport of a diagnostic agent across a membrane of a living cell. The method comprises contacting a membrane of a living cell with a complex formed between said diagnostic agent and ligands selected from biotin or biotin receptor-binding analogs of biotin, folate or folate receptor-binding analogs of folate, riboflavin or riboflavin receptor-binding analogs of riboflavin to initiate receptor mediated transmembrane transport of the ligand complex. The method is used for imaging tissues *in vivo*.

COMPOSITION AND METHOD FOR TUMOR IMAGING

This invention was made with Government support under Grant 89-45-DCB-88-11465, awarded by the National Science Foundation and Grant R01-CA46909 awarded by the National Cancer Institute. The Government has certain rights in the invention.

Field of the Invention

This invention relates to a method for enhancing transmembrane transport of exogenous molecules. More particularly, the use of nutrient receptors, including biotin or folate receptors, and the respective associated receptor mediated endocytotic mechanism associated with such receptors, is utilized to enhance the efficiency of cellular uptake of diagnostic imaging agents.

Background and Summary of the Invention

Transmembrane transport of nutrient molecules is a critical cellular function. Because practitioners have recognized the importance of transmembrane transport to many areas of medical and biological science, including drug therapy and gene transfer, there has been significant research efforts directed to the understanding and application of such processes. Thus, for example, transmembrane delivery of nucleic acids has been encouraged through the use of protein carriers, antibody carriers, liposomal delivery systems, electroporation, direct injection, cell fusion, viral carriers, osmotic shock, and calcium-phosphate mediated transformation. However, many of those techniques are limited both by the types of cells in which transmembrane transport is enabled and by the conditions of use for successful transmembrane transport of exogenous molecular species. Further, many of these known

techniques are limited in the type and size of exogenous molecule that can be transported across a membrane without loss of bioactivity.

One method for transmembrane delivery of exogenous molecules having a wide applicability is based on the mechanism of receptor mediated endocytotic activity. Unlike many other methods, receptor mediated endocytotic activity can be used successfully both *in vivo* and *in vitro*. Receptor mediated endocytosis involves the movement of ligands bound to membrane receptors into the interior of an area bounded by the membrane through invagination of the membrane. The process is initiated or activated by the binding of a receptor specific ligand to the receptor. Many receptor mediated endocytotic systems have been characterized, including those recognizing galactose, mannose, mannose 6-phosphate, transferrin, asialoglycoprotein, transcobalamin (vitamin B_{12}), -2 macroglobulins, insulin, and other peptide growth factors such as epidermal growth factor (EGF).

Receptor mediated endocytotic activity has been utilized for delivering exogenous molecules such as proteins and nucleic acids to cells. Generally, a specified ligand is chemically conjugated by covalent, ionic or hydrogen bonding to an exogenous molecule of interest, (i.e., the exogenous compound) forming a conjugate molecule having a moiety (the ligand portion) that is still recognized in the conjugate by a target receptor. Using this technique the phototoxic protein psoralen has been conjugated to insulin and internalized by the insulin receptor endocytotic pathway (Gasparro, <u>Biochem. Biophys. Res. Comm</u>. 141(2), pp. 502-509, Dec. 15, 1986); the hepatocyte specific receptor for galactose terminal asialoglycoproteins has been utilized for the hepatocyte-specific transmembrane delivery of asialoorosomucoid-poly-L-lysine non-covalently complexed to

a DNA plasmid (Wu, G.Y., J. Biol. Chem., 262(10), pp. 4429-4432, 1987); the cell receptor for epidermal growth factor has been utilized to deliver polynucleotides covalently linked to EGF to the cell interior (Myers, European Patent Application 86810614.7, published June 6, 1988); the intestinally situated cellular receptor for the organometallic vitamin B_{12}-intrinsic factor complex has been used to mediate delivery to the circulatory system of a vertebrate host a drug, hormone, bioactive peptide or immunogen complexed with vitamin B_{12} and delivered to the intestine through oral administration (Russell-Jones et al., European patent Application 86307849.9, published April 29, 1987); the mannose-6-phosphate receptor has been used to deliver low density lipoproteins to cells (Murray, G. J. and Neville, D.M., Jr., J. Bio. Chem, Vol. 255 (24), pp. 1194-11948, 1980); the cholera toxin binding subunit receptor has been used to deliver insulin to cells lacking insulin receptors (Roth and Maddox, J. Cell. Phys. Vol. 115, p. 151, 1983); and the human chorionic gonadotropin receptor has been employed to deliver a ricin a-chain coupled to HCG to cells with the appropriate HCG receptor in order to kill the cells (Oeltmann and Heath, J. Biol. Chem, Vol. 254, p. 1028 (1979)).

The method of the present invention enhances the transmembrane transport of an exogenous molecule across a membrane having biotin or folate receptors that initiate transmembrane transport of receptor bound species. The method takes advantage of (1) the location and multiplicity of biotin and folate receptors on the membrane surfaces of most cells and (2) the associated receptor mediated transmembrane processes. Performance of the method involves formation of a complex between a ligand selected from biotin or other biotin receptor-binding compounds, and/or folic acid or other folate receptor-binding compounds, and an exogenous molecule. A cell membrane

bearing biotin or folate receptors is contacted with this complex, thereby initiating receptor mediated transmembrane transport of the complex. The complex is allowed to contact the membrane surface bearing the corresponding receptors for a time sufficient to initiate and permit transmembrane transport of the complex. The transmembrane transport of exogenous molecules including proteins and polynucleotides has been promoted in plant, mammalian, and bacterial cells.

In one embodiment of this invention, the target receptor for the method of the present invention is the biotin receptor. Biotin is a necessary cellular nutrient that has been found to be preferentially bound by biotin receptor proteins associated with cellular membranes. Commercially available reagents are used to form a covalent complex between biotin and polynucleotides, proteins, or other desired exogenous molecules. According to one preferred embodiment of the present invention, a biotin/exogenous molecule complex is brought into contact with a membrane having associated biotin receptors for a time sufficient to allow binding of the biotin moiety of the complex to a corresponding biotin receptor in the membrane. This binding triggers the initiation of cellular processes that result in transmembrane transport of the complex.

In an alternate but equally preferred embodiment of this invention, folate receptors are targeted to enhance cellular uptake of exogenous molecules. Folate binding receptors are found in most types of cells, and they have been demonstrated to bind and trigger cellular internalization of folates. Thus, folic acid and other art-recognized folate receptor-binding ligands can be chemically bonded to polynucleotides, proteins, or other desired exogenous molecules using art-recognized coupling techniques to provide a folate receptor-binding complex

which is readily endocytosed into living cells. In accordance with this embodiment of the present invention, a folate/exogenous molecule complex is brought into contact with a membrane having associated folate receptors for a time sufficient to allow binding of the folate moiety of the complex to a corresponding folate receptor. Folate receptor-binding triggers the initiation of cellular processes that result in transmembrane transport of the complex.

The methods of this invention are particularly useful for increasing the internalization efficiency (cellular uptake) of exogenous molecules that are normally resistant to cellular internalization. Proteins and polynucleotides previously recognized as difficult to move across cell membranes can be internalized by a cell through application of the method of the present invention. For example, transformation of target cell lines resulting in expression of a protein product has been accomplished by coupling the desired polynucleotide to either biotin or folates, and contacting the cells with the resulting complex for a time sufficient to promote cellular internalization. In one case, a DNA plasmid containing a gene sequence coding for chloramphenicol acetyltransferase (CAT), was biotinylated and transported into E. coli via a biotin receptor mediated endocytotic pathway and expressed. Similar examples of transformation or transection have been noted for biotin or folate linked nucleic acids in mammalian systems, prokaryotic systems, and plants. The use of biotin and folate complexes to enhance cellular uptake of complexed exogenous molecules has been demonstrated *in vivo* and *in vitro*.

Brief Description of the Drawings

Fig. 1 illustrates the structures of chelators useful for forming the folate-radionuclide complexes of the

present invention. 〰 Represents an organic "spacer" that can be a saturated or unsaturated hydrocarbon and that possibly incorporates other heteroatoms (e.g., O, N, or S). The substituent(s) "X" represents (represent) one or more functional groups on the aromatic rings that can be: alkyl, alkoxy, alkyl ether, amine, amide, ester, carboxylate, or alcohol sidechains; additional substituted or unsubstituted aromatic rings; halogen substituents; or a hydrogen atom. The substituents "R" can be hydrogen atoms, alkyl groups, and/or substituted or unsubstituted aromatic rings.

Fig. 2 illustrates the measured mouse serum folate levels as a function of time following initiation of the folate-deficient diet.

Fig. 3 is an illustration of a deferoxamine-folate conjugate which can be radiolabeled with ^{67}Ga.

Fig. 4 is a graphic representation of the cellular uptake by BHK cells of ^{125}I labeled ribonuclease/riboflavin conjugates. At varying timepoints, the cells were washed 5X in saline, and counted in a gamma counter.

Fig. 5 illustrates the biodistribution of ^{125}I-BSA-riboflavin conjugate following administration to Wistar female rats. The cross-hatched bars represent the bovine serum albumin (BSA) content of tissues from rats treated with the ^{125}I-BSA-riboflavin conjugated samples, while the open bars represent the bovine serum albumin (BSA) content of tissues from rats treated with ^{125}I-BSA.

Fig. 6 illustrates the cellular internalization of thiamin-BSA and riboflavin-BSA complexes by cultured A549 cells.

Fig. 7 is a graphic representation of the time dependant uptake of BSA and BSA-thiamin complexes by KB cells.

Fig. 8 illustrates the percent injected dose of ^{67}Ga-radiotracer (^{67}Ga-citrate, ^{67}Ga-deferoxamine, and ^{67}Ga-deferoxamine-folate) per gram tumor. Each bar represents the data from one animal. Group 1 was administered ^{67}Ga-deferoxamine-folate; Group 2 was administered ^{67}Ga-deferoxamine-folate to mice maintained on a high folate diet; Group 3 was administered folic acid (approximately 2.4 mg) prior to administration of ^{67}Ga-deferoxamine-folate; Group 4 was administered ^{67}Ga-deferoxamine-folate with a chase dose of folate one hour prior to sacrifice; Group 5 was administered ^{67}Ga-deferoxamine; Group 6 was administered ^{67}Ga-citrate.

Fig. 9 illustrates the tumor to blood ratios (% of injected dose per gram wet weight) at 4-4.5 hours post-injection for ^{67}Ga-radiotracers: ^{67}Ga-citrate, ^{67}Ga-deferoxamine, and ^{67}Ga-deferoxamine-folate. Each bar represents data from one animal. Group 1 was administered ^{67}Ga-deferoxamine-folate; Group 2 was administered ^{67}Ga-deferoxamine-folate to mice maintained on a high folate diet; Group 3 was administered folic acid (approximately 2.4 mg) prior to administration of ^{67}Ga-deferoxamine-folate; Group 4 was administered ^{67}Ga-deferoxamine-folate with a chase dose of folate one hour prior to sacrifice; Group 5 was administered ^{67}Ga-deferoxamine; Group 6 was administered ^{67}Ga-citrate.

Fig. 10 is an illustration of a DTPA-folate conjugate which can be radiolabeled with ^{111}In.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with one embodiment of this invention, there is provided a method for enhancing transport of an exogenous molecule across a membrane of a living cell. The method comprises the step of contacting the membrane with the exogenous molecule complexed with a ligand selected from the group consisting of biotin, biotin

receptor-binding analogs of biotin, and other biotin receptor-binding ligands, for a time sufficient to permit transmembrane transport of said ligand complex. In a second embodiment, there is provided a method for enhancing transport of an exogenous molecule across a membrane of a living cell, comprising the step of contacting the membrane with the exogenous molecule complexed with a ligand selected from the group consisting of folic acid, folate receptor-binding analogs of folic acid, and other folate receptor-binding ligands, for a time sufficient to permit transmembrane transport of said ligand complex.

The method of the present invention is effective in all living cells that have biotin and/or folate receptors associated with their cellular membranes. The membrane can define an intracellular volume such as the endoplasmic reticulum or other organelles such as mitochondria, or alternatively, the membrane can define the boundary of the cell.

Living cells which can serve as the target for the method of this invention include prokaryotes and eukaryotes, including yeasts, plant cells and animal cells. The present method can be used to modify cellular function of living cells *in vitro*, i.e., in cell culture, or *in vivo*, where the cells form part of or otherwise exist in plant tissue or animal tissue. Thus the cells can form, for example, the roots, stalks or leaves of growing plants and the present method can be performed on such plant cells in any manner which promotes contact of the exogenous molecule/folate or biotin complex with the targeted cells having the requisite receptors. Alternatively, the target cells can form part of the tissue in an animal. Thus the target cells can include, for example, the cells lining the alimentary canal, such as the oral and pharyngeal mucosa, the cells forming the villi of the small intestine, or the cells lining the large intestine. Such cells of the

alimentary canal can be targeted in accordance with this invention by oral administration of a composition comprising an exogenous molecule complexed with folates or biotin or their receptor-binding analogs. Similarly, cells lining the respiratory system (nasal passages/lungs) of an animal can be targeted by inhalation of the present complexes; dermal/epidermal cells and cells of the vagina and rectum can be targeted by topical application of the present complexes; and cells of internal organs including cells of the placenta and the so-called blood/brain barrier can be targeted particularly by parenteral administration of the present complexes. Pharmaceutical formulations for therapeutic use in accordance with this invention containing effective amounts of the presently described folate and biotin complexes, in admixture with art-recognized excipients appropriate to the contemplated route of administration are within the scope of this invention.

Since not all natural cell membranes possess biologically active biotin or folate receptors, practice of the method of this invention *in vitro* on a particular cell line can involve altering or otherwise modifying that cell line first to ensure the presence of biologically active biotin or folate receptors. Thus, the number of biotin or folate receptors on a cell membrane can be increased by growing a cell line on biotin or folate deficient substrates to promote biotin and folate receptor production, or by expression of an inserted foreign gene for the protein or apoprotein corresponding to the biotin or folate receptor.

The present invention is utilized to enhance the cellular uptake of exogenous molecules, in particular those molecules capable of modulating or otherwise modifying cell function, including pharmaceutically active compounds or diagnostic agents. Suitable exogenous molecules can

include, but are not limited to: peptides, oligopeptides, proteins, apoproteins, glycoproteins, antigens and antibodies thereto, haptens and antibodies thereto, receptors and other membrane proteins, retro-inverso oligopeptides, protein analogs in which at least one non-peptide linkage replaces a peptide linkage, enzymes, coenzymes, enzyme inhibitors, amino acids and their derivatives, hormones, lipids, phospholipids, liposomes; toxins such as aflatoxin, digoxin, xanthotoxin, rubratoxin; antibiotics such as cephalosporins, penicillin, and erythromycin; analgesics such as aspirin, ibuprofen, and acetaminophen, bronchodilators such theophylline and albuterol; beta-blockers such as propranolol, metoprolol, atenolol, labetolol, timolol, penbutolol, and pindolol; antimicrobial agents such as those described above and ciprofloxacin, cinoxacin, and norfloxacin; antihypertensive agents such as clonidine, methyldopa, prazosin, verapamil, nifedipine, captopril, and enalapril; cardiovascular agents including antiarrhythmics, cardiac glycosides, antianginals and vasodilators; central nervous system agents including stimulants, psychotropics, antimanics, and depressants; antiviral agents; antihistamines such as chlorpheniramine and brompheniramine; cancer drugs including chemotherapeutic agents; tranquilizers such as diazepam, chordiazepoxide, oxazepam, alprazolam, and triazolam; anti-depressants such as fluoxetine, amitriptyline, nortriptyline, and imipramine; H-2 antagonists such as nizatidine, cimetidine, famotidine, and ranitidine; anticonvulsants; antinauseants; prostaglandins; muscle relaxants; anti-inflammatory substances; stimulants; decongestants; antiemetics; diuretics; antispasmodics; antiasthmatics; anti-Parkinson agents; expectorants; cough suppressants; mucolytics; vitamins; and mineral and nutritional additives. Other molecules include nucleotides; oligonucleotides; polynucleotides; and their

art-recognized and biologically functional analogs and derivatives including, for example; methylated polynucleotides and nucleotide analogs having phosphorothioate linkages; plasmids, cosmids, artificial chromosomes, other nucleic acid vectors; antisense polynucleotides including those substantially complementary to at least one endogenous nucleic acid or those having sequences with a sense opposed to at least portions of selected viral or retroviral genomes; promoters; enhancers; inhibitors; other ligands for regulating gene transcription and translation, and any other biologically active molecule that can form a complex with biotin or folate, or analogs thereof, by direct conjugation of the exogenous molecule with biotin or biotin analog or folate or folate analog through a hydrogen, ionic, or covalent bonding. Also in accordance with this invention is the use of indirect means for associating the exogenous molecule with biotin or folate, or analogs thereof to form liquid complexes, such as by connection through intermediary linkers, spacer arms, bridging molecules, or liposome entrapment, all of which can act to associate the biotin or biotin analog or folate or folate analog with the exogenous molecule of interest. Both direct and indirect means for associating the ligand and the exogenous molecule must not prevent the binding of the ligand held in association with the exogenous molecule to its respective ligand receptor on the cell membrane for operation of the method of the present invention.

Generally, any manner of forming a complex between an exogenous molecule of interest and a ligand capable of triggering receptor mediated endocytosis can be utilized in accordance with the present invention. This can include covalent, ionic, or hydrogen bonding of the ligand to the exogenous molecule, either directly or indirectly via a linking group. The complex is typically formed by covalent bonding of the receptor-activating

moiety to the exogenous molecule through the formation of amide, ester or imino bonds between acid, aldehyde, hydroxy, amino, or hydrazo groups on the respective components of the complex. Art-recognized biologically labile covalent linkages such as imino bonds (-C=N-) and so-called "active" esters having the linkage $-COOCH_2O$ or $-COOCH(CH_3)O$ are preferred, especially where the exogenous molecule is found to have reduced functionality in the complexed form. Hydrogen bonding, e.g., that occurring between complementary strands of nucleic acids, can also be used for complex formation. Thus a biotinylated or folated oligonucleotide complementary to at least a portion of a nucleic acid to be delivered to a cell in accordance with this invention can be hybridized with said nucleic acid and the hybrid (complex) used per this invention to enhance delivery of the nucleic acid into cells.

Because of the ready availability of biotinylating reagents and biotinylating methods suitable for use with peptides, proteins, oligonucleotides, polynucleotides, lipids, phospholipids, carbohydrates, liposomes or other lipid vesicles, lower molecular weight therapeutic agents, bioactive compounds, and carriers for therapeutic agents, biotin is a preferred complex forming ligand for use in carrying out this invention. Generally, the biotin/exogenous molecule complex is formed by covalently binding biotin or a biotin derivative to the exogenous molecule of interest. Transmembrane transport via the biotin/biotin receptor pathway is also preferred because biotin is a necessary nutrient for a wide variety of cells, and biotin receptors that mediate endocytotic activity have been identified in mammalian, plant, and bacterial cells.

Formation of a complex between biotin and an exogenous molecule of interest is readily accomplished. Biotin and its analogs can be easily conjugated to proteins

by activating the carboxyl group of biotin, thereby making it reactive with the free amino groups of the proteins to form a covalent amide linking bond. A biotinylating reagent such as D-biotin-N-hydroxy-succinimide ester or biotinyl-p-nitrophenyl ester can be used. The activated ester reacts under mild conditions with amino groups to incorporate a biotin residue into the desired molecule. The procedure to be followed for biotinylating macromolecules using D-biotin-N-hydroxy-succinimide ester is well known in the art (Hofmann et al., J. Am. Chem. Soc. 100, 3585-3590 (1978)). Procedures suitable for biotinylating an exogenous molecule using biotinyl-p-nitrophenyl ester as a biotinylating reagent are also well known in the art (Bodanszk et al., J. Am. Chem. Soc. 99, 235 (1977)). Other reagents such as D-biotinyl- -aminocaproic acid N-hydroxy-succinimide ester in which -aminocaproic acid serves as a spacer link to reduce steric hindrance can also be used for the purposes of the present invention.

Oligonucleotides and polynucleotides can also be biotinylated using both indirect and direct methods. Indirect methods include end-labeling of a polynucleotide with a biotinylated nucleotide, or nick translation that incorporates biotinylated nucleotides. Nick translation or end labeling of DNA can be accomplished using methods described in Maniatis et al., Molecular Cloning: A Laboratory Manual, pp. 109-116, Cold Spring Harbor Press (1982). Direct methods are those procedures in which biotin is directly attached to a target polynucleotide using a biotinylating reagent. Photoactivatible reagents such as the acetate salt of N-(4-azido-2-nitrophenyl)-N-(3-biotinylaminopropyl)-N-methy 1-1,3-propanediamine (photobiotin) can be used to biotinylate DNA according to the method of Forster et al., Nuc. Acids Res. 13:745-761. An alternative method uses a

biotin hydrazide reagent in a bisulfite catalyzed reaction capable of transamination of nucleotide bases such as cytidine according to the method described by Reisfeld et al., B.B.R.C. 142:519-526 (1988). This method simply requires a 24 hour incubation of DNA or RNA with biotin hydrazide at 10mg/ml in an acetate buffer, pH 4.5, containing 1 M bisulfite. Biotin hydrazide can also be used to biotinylate carbohydrates or other exogenous molecules containing a free aldehyde.

Biotin analogs such as biocytin, biotin sulfoxide, oxybiotin and other biotin receptor-binding compounds are liquids that may also be used as suitable complexing agents to promote the transmembrane transport of exogenous molecules in accordance with this invention. Other compounds capable of binding to biotin receptors to initiate receptor mediated endocytotic transport of the complex are also contemplated. Such can include other receptor-binding ligands such as, for example, anti-idiotypic antibodies to the biotin receptor. An exogenous molecule complexed with an anti-idiotypic antibody to a biotin receptor could be used to trigger transmembrane transport of the complex in accordance with the present invention.

Folate receptors that mediate endocytotic activity have previously been identified in bacterial cells (Kumar et al., J. Biol. Chem., 262, 7171-79 (1987)). Folic acid, folinic acid, pteropolyglutamic acid, and folate receptor-binding pteridines such as tetrahydropterins, dihydrofolates, tetrahydrofolates, and their deaza and dideaza analogs are preferred complex-forming ligands used in accordance with a second embodiment of this invention. The terms "deaza" and "dideaza" analogs refers to the art recognized analogs having a carbon atom substituted for one or two nitrogen atoms in the naturally occurring folic acid structure. For example, the deaza analogs include the

1-deaza, 3-deaza, 5-deaza, 8-deaza, and 10-deaza analogs. The dideaza analogs include, for example, 1,5 dideaza, 5,10-dideaza, 8,10-dideaza, and 5,8-dideaza analogs. The foregoing folic acid derivatives are conventionally termed "folates", reflecting their capacity to bind with folate-receptors, and such ligands when complexed with exogenous molecules are effective to enhance transmembrane transport. Other folates useful as complex forming ligands for this invention are the folate receptor-binding analogs aminopterin, amethopterin (methotrexate), N^{10}-methylfolate, 2-deamino-hydroxyfolate, deaza analogs such as 1-deazamethopterin or 3-deazamethopterin, and 3',5'-dichloro-4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid (dichloromethotrexate). Other suitable ligands capable of binding to folate receptors to initiate receptor mediated endocytotic transport of the complex include anti-idiotypic antibodies to the folate receptor. An exogenous molecule in complex with an anti-idiotypic antibody to a folate receptor is used to trigger transmembrane transport of the complex in accordance with the present invention.

Folated ligands can be complexed with the exogenous molecules hereinbefore defined using art-recognized covalent coupling techniques identical to or closely paralleling those referenced above for the biotinylate ligand complexes. Thus, for example, a carboxylic acid on the folate moiety or on the exogenous molecule can be activated using, for example, carbonyldiimidazole or standard carbodimide coupling reagents such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and thereafter reacted with the other component of the complex having at least one nucleophilic group, viz hydroxy, amino, hydrazo, or thiol, to form the respective complex coupled through an ester, amide, or thioester bond. Thus

complexes can be readily formed between folate ligands and peptides, proteins, nucleic acids, including both RNA and DNA, phosphorodithioate analogs of nucleic acids, oligonucleotides, polynucleotides, lipids and lipid vesicles, phospholipids, carbohydrates and like exogenous molecules capable of modifying cell function. The ligand complexes enable rapid, efficient delivery of the cell function-modifying moiety through cellular membranes and into the cell.

It is contemplated that both folate and biotinylate-receptor binding ligands can be used advantageously in combination to deliver exogenous molecules through cell membranes. Thus, for example, an exogenous molecule can be multiply conjugated with both folate and biotinylate ligands to enhance opportunity for binding with the respective cell membrane receptors. Alternatively, independent portions of a dose of an exogenous compound can be biotinylated and folate-coupled, respectively, and the portions of the resulting complexes can subsequently be combined to provide a mixture of ligand complexes for modification of cell function.

Receptor mediated cellular uptake of biotinylated or folate-derivatized polynucleotides provides a convenient, efficient mechanism for transformation of cells. The method is particularly valuable for cell transformation because it is applicable even to cell types, such as plant cells, which are normally resistant to standard transformation techniques. Delivery of foreign genes to the cell cytoplasm can be accomplished with high efficiency using the present invention. Once delivered through the cell membrane to the cell interior, foreign genes can be expressed to produce a desired protein. In addition, other nucleic acids can be introduced, for example, an antisense-RNA sequence capable of binding interference with endogenous messenger RNA.

Artificially generated phospholipid vesicles have been used as carriers for introducing membrane-impermeable substances into cells, as instruments for altering lipid composition of membranes in intact cells, and as inducers of cell fusion. Liposome/cell membrane interaction is potentiated in accordance with one application of the method of this invention by contacting the cell membrane with a liposome containing the exogenous molecule and bearing ligands on its membrane contacting surface. For example, liposome-forming phospholipids can be biotinylated or folate-conjugated through, for example, headgroup functional groups such as hydroxy and amino groups. The resulting phospholipid/ligand complex is then used itself or in combination with unmodified phospholipids to form liposomes containing exogenous molecules capable of modulating or otherwise modifying cell function. The resulting liposomes, again formed in whole or in part from the phospholipid/biotin or folate complex, present biotin or folate receptor-binding groups to the cell surface, triggering the receptor mediated endocytosis mechanism, thereby promoting delivery of the liposome-contained substances into the cell. One readily available phospholipid that can be used in accordance with the above-described method is phosphatidylethanolamine. That phospholipid can be conveniently complexed using art-recognized procedures with either biotin, biotin analogs or folate-receptor-binding ligands to form a phospholipid/ligand complex. The receptor-binding complex can be combined with other phospholipids, for example, phosphatidylcholine and that mixture can be used to form liposomes containing biologically active substances for delivery of those biologically active substances to cells.

It is further contemplated in accordance with this invention that other cell nutrients for which there exists receptors and associated receptor mediated

endocytotic uptake could serve as ligands for forming complexes with exogenous molecules to enhance their cellular uptake. Among nutrients believed to trigger receptor mediated endocytosis and having application in accordance with the presently disclosed method are carnitine, inositol, lipoic acid, niacin, pantothenic acid, riboflavin, thiamin, pyridoxal, and ascorbic acid, and the lipid soluble vitamins A, D, E and K. These non-organometallic nutrients, and their analogs and derivatives thereof, constitute ligands that can be coupled with exogenous molecules to form ligand complexes for contact with cell membranes following the same procedures described hereinabove for biotin and folate. These foregoing nutrients are generally required nutrients for mammalian cells. Exogenous molecules coupled with the foregoing non-organometallic nutrients can be used to deliver effective amounts of therapeutic agents or pharmaceutically active agents such as previously described through parenteral or oral routes of administration to human or animal hosts.

In accordance with one embodiment of the present invention, the exogenous molecule comprises a diagnostic agent that is complexed with a ligand to enhance transport of the diagnostic agent across a membrane of a living cell. The ligand is selected from the group consisting of biotin or biotin receptor-binding analogs of biotin, folate or folate receptor-binding analogs of folate, riboflavin or riboflavin receptor-binding analogs of riboflavin, and thiamin or thiamin receptor-binding analogs of thiamin. Complexing a vitamin ligand to a diagnostic agent allows the diagnostic agent to be targeted, upon administration to an animal, to tissues that possess membrane-bound receptors for the vitamin ligand. This results in an enhanced concentration of the diagnostic agent at the target tissues

and provides rapid clearance of the diagnostic agent from non-target tissue.

Diagnostic agents suitable for use in the present invention include any compound that is capable of being detected *in vivo* after administration to a multicellular organism. Preferred compounds include electron dense materials, magnetic resonance imaging agents and radiopharmaceuticals.

The ligand can be complexed to the diagnostic agent by covalent, ionic or hydrogen bonding either directly or indirectly through a linking group. In one embodiment the diagnostic agent is contained in a liposome, wherein the liposome comprises liposome-forming phospholipds, at least a portion of which are covalently bound through their headgroups to the ligand.

In one embodiment ligands selected from the group consisting of biotin or biotin receptor-binding analogs of biotin, folate or folate receptor-binding analogs of folate, riboflavin or riboflavin receptor-binding analogs of riboflavin, and thiamin or thiamin receptor-binding analogs of thiamin, are coupled to radionuclides and used for diagnostic imaging. Radionuclide suitable for diagnostic imaging include radioisotopes of gallium, indium, copper, technetium and rhenium, including isotopes ^{111}In, ^{99m}Tc, ^{64}Cu, ^{67}Cu, ^{67}Ga or ^{68}Ga. These radionuclides can be conjugated to a vitamin ligand through a chelating linking group. The chemical structure of the chelating agent is not critical provided that it have the requisite affinity for the radionuclide cation. Suitable chelating agents for use in accordance with the present invention include the chelates shown in Fig. 1 as well as tetraazacyclotetradecanetetraacetate (TETA).

In one embodiment of the present invention a ligand-radiopharmaceutical complex is used to image tumor cells. In particular, folic acid-radionuclide complexes

have been used to image tumor cells. Folic acid is an essential dietary vitamin needed by all eukaryotic cells for DNA synthesis and carbon metabolism. Folic acid primarily enters cells through facilitated transport by a membrane transport protein ($K_m = 1.5 \times 10^{-6}$ M for folic acid), however, some cells also possess a membrane-bound folate-binding-protein receptor (FBP) that secondarily allows folate uptake *via* receptor mediated endocytosis ($K_a = 5 \times 10^{-10}$ M for folate). When folate is covalently bonded, directly or indirectly through a linking group, to a diagnostic agent *via* its gamma-carboxylate, the folate fragment ceases to be recognized by the facilitated transport system, but can still be recognized by the FBP receptor. Thus, such folate-conjugates are selectively concentrated by cells that express the membrane FBP receptor.

A number of tumor cell types (*e.g.*, breast, ovarian, cervical, colorectal, renal, and nasopharyngeal) are known to overexpress FBP receptors. Conjugation of diagnostic agents, such as radiopharmaceuticals, to the gamma-carboxylate of folate enhances the selective uptake of these complexes by tumor cells allowing for more rapid and sensitive imaging of tumors.

^{125}I labeled ribonuclease-folate was used to evaluate radiotracer delivery to tumor cells in athymic mice maintained on a folate-free diet (to regulate serum folate concentration closer to levels found in normal human serum). Tumor cells were implanted in athymic mice by subcutaneous injection of 2×10^6 human KB cells into the shoulder of the mice. The mice were administered the ^{125}I labeled ribonuclease-folate conjugate intravenously *via* the femoral vein twenty days after subcutaneous injection the human KB cells. As a control, tumor bearing athymic mice were injected with ^{125}I labeled ribonuclease (lacking folate). The biodistribution of each agent, calculated as

a percentage of the injected dose per gram of tissue, is shown in tables 1 (^{125}I-ribonuclease-folate) and 2 (^{125}I-ribonuclease). Some tumor selectively is apparent based on comparison of the tumor uptake and tumor/blood ratios for ^{125}I-ribonuclease-folate and ^{125}I-ribonuclease. However, this level of selectivity is not sufficient to afford clinical utility, due to poor tumor contrast with other non-target tissues.

TABLE 1

Biodistribution of ^{125}I-RNase-Folate Conjugate Following I.V. Administration to Male Athymic Mice (Folate-free diet) With KB Tumors

	Percentage of Injected Dose per Gram		
	1 hour	4 hours	24 hours
Blood	5.34 ± 1.07	2.30 ± 0.62	0.04 ± 0.01
Heart	2.01 ± 0.22	0.93 ± 0.39	0.024 ± 0.006
Lungs	4.19 ± 0.61	1.97 ± 0.77	0.04 ± 0.01
Liver	9.78 ± 1.11	3.29 ± 0.77	0.38 ± 0.03
Spleen	9.94 ± 1.37	2.78 ± 0.73	0.23 ± 0.04
Kidney	16.08 ± 2.76	5.11 ± 1.27	0.72 ± 0.07
Brain	0.29 ± 0.04	0.19 ± 0.14	0.007 ± 0.001
Muscle	1.73 ± 0.32	0.98 ± 0.54	0.013 ± 0.002
Testes	1.50 ± 0.18	*1.0 ± 0.15	0.021 ± 0.004
Bone	3.20 ± 0.23	1.17 ± 0.28	0.09 ± 0.03
Thyroid	-	-	-
Tumor	5.35 ± 0.54	2.74 ± 0.51	0.41 ± 0.02
Stomach	21.07 ± 2.57	26.45 ± 8.05	0.26 ± 0.14
Intestines	2.03 ± 0.28	0.95 ± 0.16	0.05 ± 0.006
Tumor/Blood	*1.02 ± 0.16*	*1.21 ± 0.17*	*11.88 ± 3.71*
Tumor/Muscle	*3.14 ± 0.43*	*3.35 ± 1.55*	*32.6 ± 6.7*
n	4	3	3

*n=2

TABLE 2

Biodistribution of ^{125}I-RNase (Control) Following I.V. Administration to Male Athymic Mice (Folate-free diet) With KB Tumors

	Percentage of Injected Dose per Gram		
	1 hour	4 hours	24 hours
Blood	4.99 ± 1.22	1.06 ± 7.31	0.06 ± 0.01
Heart	1.72 ± 0.41	0.40 ± 0.09	0.026 ± 0.007
Lungs	3.65 ± 0.81	0.79 ± 0.21	0.047 ± 0.012
Liver	1.83 ± 0.72	0.42 ± 0.13	0.91 ± 1.73
Spleen	2.12 ± 0.50	0.53 ± 0.14	0.023 ± 0.006
Kidney	24.3 ± 5.4	5.84 ± 0.18	1.66 ± 0.26
Brain	0.19 ± 0.06	0.06 ± 0.004	0.0055 ± 0.0006
Muscle	1.27 ± 0.24	0.35 ± 0.10	0.021 ± 0.005
Testes	1.79 ± 1.18	0.52 ± 0.05	0.017 ± 0.005
Bone	1.89 ± 0.31	0.55 ± 0.16	0.07 ± 0.05
Thyroid	-	-	-
Tumor	3.31 ± 1.75	0.87 ± 0.26	0.038 ± 0.012
Stomach	21.37 ± 9.72	9.80 ± 4.24	0.21 ± 0.14
Intestines	1.92 ± 0.26	0.56 ± 0.12	0.06 ± 0.03
Tumor/Blood	*0.07 ± 0.38*	*0.82 ± 0.07*	*0.64 ± 0.09*
Tumor/Muscle	*2.7 ± 1.4*	*2.51 ± 0.54*	*1.85 ± 0.37*
n	3	3	4

Surprisingly, low molecular weight diagnostic agents when complexed to folate and administered to animals were found to produce significantly higher tumor to normal tissue biodistribution ratios. Since the uptake of folate conjugates is mediated by receptor mediated endocytotic mechanisms, and these mechanisms are generally capable of internalizing large macromolecules, one would not expect that lower molecular weight folate conjugates would be more effective than high molecular weight folate conjugates. In particular radionuclides complexed to folate via a chelating agent show a high affinity for FBP receptors and thus are excellent compounds for diagnostic imaging. In accordance with the present invention a radionuclide folate conjugate of the general formula:

$$V\text{-}Y\bullet M$$

wherein V = folate or folate receptor-binding analogs of folate;

Y = a chelating agent covalently bound to V; and

M = a radionuclide ion chelated with Y;

is used to image tumor cells *in vivo*.

In particular, gallium labeled folate complexes have been used *in vivo* in mice to image tumors, and these complexes demonstrate a particularly high affinity for tumor cells. Localization of tumor masses as small as a few milligrams in size should be readily visible and may allow their removal before further metastases can occur.

To create an animal model appropriate for evaluation of FBP-receptor targeting *in vivo*, athymic mice were implanted subcutaneously with *ca.* 4 x 10^6 cells of the human KB line. Since normal mouse food contains a high concentration of folic acid (6 mg/kg chow), the animals used in the tumor-targeting studies were generally maintained on folate-free diet to regulate serum folate

concentration closer to the 4-6 $\mu g/L$ range of normal human serum. Fig. 2 shows the measured mouse serum folate levels as a function of time following initiation of the folate-deficient diet.

To test the tumor-cell-selective uptake of metal-labeled radiopharmaceuticals *in vivo*, ~180 μCi of ^{67}Ga-deferoxamine-folate conjugate (See Fig. 3) was administered to two tumor-bearing athymic mice. Tumors were generated in athymic mice by subcutaneous injecting human KB cells into the dorsal-lateral region of the mice according to procedures familiar to those of ordinary skill in the art. After establishment of tumors in the mice, ~180 μCi of ^{67}Ga-deferoxamine-folate conjugate was administered intravenously. Approximately 45 hours post-injection, gamma images were obtained and the tissue distribution of ^{67}Ga quantitated. Upon dissection, the tumors from these two animals were found to have masses of 29.6 and 8 mg, while the total body mass of these animals was 19.3 and 22.6 g, respectively. Despite the small size and sub-optimal positioning of these tumors relative to the kidneys, the 29.6 mg tumor was readily detected by gamma scintigraphy. At sacrifice the 29.6 mg tumor was found to contain 3.3% of the injected dose per gram of tumor.

To better define the ability of ^{67}Ga-DF-folate to target tumor cells *in vivo* and to confirm the role of the FBP receptor in determining conjugate tumor uptake, a series of 17 additional athymic tumor bearing mice were studied as described in Example 26. The ^{67}Ga-DF-folate complex was delivered intravenously to the mice and the resulting tissue distributions of the gallium-deferoxamine-folate complex are shown in Table 3. The relatively low molecular weight ^{67}Ga-deferoxamine-folate conjugates have significantly higher absolute tumor uptake of the imaging agent and much better tumor to non-target tissue contrast than those obtained with ^{125}I labeled ribonuclease-folate

complexes. At 4 hours post-injection, tumor uptake of the ^{67}Ga-deferoxamine-folate conjugate was 5.2 ± 1.5% of the injected dose per gram, while ^{125}I-ribonuclease-folate yielded only 2.7 ± 0.5% of the injected dose per gram. The corresponding tumor/blood ratios are 409 ± 195 for ^{67}Ga-deferoxamine-folate and 1.2 ± 0.2 for ^{125}I-ribonuclease-folate and the corresponding tumor/muscle ratios are 124 ± 47 for ^{67}Ga-deferoxamine-folate and 3.4 ± 1.6 for ^{125}I-ribonuclease-folate. Through the use of a gamma camera, 8 mm tumors were easily imaged *in vivo* using the ^{67}Ga-deferoxamine-folate complex.

It is anticipated that other low molecular weight radiopharmaceuticals can be coupled to the gamma-carboxylate of folate for imaging of tumor cells. In one embodiment, a radiolabeled peptide can be complexed to folate. The peptide moiety of the folate-peptide complex can be selected from peptides/protein fragments that bind to tumor associated receptors. Peptides having an affinity for tumor associated receptors have been previously described and are known to those skilled in the art. The conjugation of such peptides to folate, either directly or indirectly through a linker, can impart additional tumor affinity to the imaging agent and thus further enhance the selectivity of the imaging complex for tumor cells.

The following examples are provided to illustrate further the method of the present invention.

<u>Example 1</u> - RAT PHEOCHROMOCYTOMA CELL UPTAKE OF BIOTIN CONJUGATED INSULIN:

Rat pheochromocytoma (PC-12) cells were obtained from America Type Culture Collection and were grown (37°C, 5% CO_2 in humidified air) attached to plastic flasks for 2 to 3 weeks until confluent in a medium of 85% RMPI 1640, 10% v/v heat inactivated horse serum, and 5% fetal calf serum containing 1% streptomycin-penicillin.

Biotin and fluorescein labeled insulin was prepared. To 1 ml of a 1 mg/ml solution of insulin protein in phosphate buffered saline was added simultaneously 100 l of a 1 mg/ml solution of fluorescein isothiocyanate (FITC) in dimethylformamide (DMF) and 100 l of a 1 mg/ml solution of N-hydroxysuccinimido biotin in dimethylsulfoxide (DMSO). The two labeling reagents were allowed to react at room temperature for 4 hours, after which the unreacted reagents were quenched with 10 l ethanolamine. The quenched reaction mixture was then dialyzed against double distilled water until unreacted fluorescein derivatives no longer dialyzed into the water. The covalent attachment of biotin and fluorescein to the desired protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot analysis.

As a control, non-biotinylated fluorescein labeled insulin was prepared. 1 ml of a 1 mg/ml solution of insulin was added 0.5 ml of a 1 mg/ml solution of fluorescein isothiocyanate (FITC) in dimethylformamide (DMF). The reaction was allowed to proceed for 4 hours in the dark at room temperature. After 4 hours the reaction was quenched with 10 l ethanolamine, and the labeled insulin solution was dialyzed against double distilled water until unreacted FITC no longer appeared in the solution.

The rat PC12 cells were grown in modified RMPI 1640 medium as a monolayer on the bottom of a culture flask. Before removing the cells, the monolayer was washed with a 20 ml portion of fresh Locke's solution. The cells were then displaced into 20 ml of the Locke's solution by gentle agitation with a stream Locke's solution. The suspended cells were pelleted by centrifugation at 10,000 x g for 10 seconds and after resuspending in Locke's solution in separate polycarbonate tubes (40ml/tube) to a final

density of 1.14 x 10^6 cells/ml, the following amounts of proteins were added to the cell suspensions: 40 g fluorescein-labeled insulin was added to the first tube, and to the control tube was added 40 g biotin-conjugated insulin labeled with fluorescein. The tubes were allowed to incubate at 37°C. At intervals of 5, 15 and 33 minutes, 0.5 ml of each cell suspension was removed and pelleted at 10,000 x g for 10 seconds. The cell pellet was washed and repelleted twice in 1 ml Locke's solution and then fixed by addition of 200 l of a 2% formalin solution in phosphate buffered saline. Thirteen microliters of the fixed cell suspension was then added to a microscope slide and viewed with the fluorescent microscope to detect internalized proteins. No evidence of internalization was noted for the fluorescein labeled insulin acting as a control. Cellular internalization was indicated for the biotinylated insulin labeled with fluorescein, with the amount internalized increasing with time.

Example 2 - RAT PHEOCHROMOCYTOMA CELL UPTAKE OF BIOTIN CONJUGATED HEMOGLOBIN:

Following the same general procedure set forth in Example 1 hemoglobin was biotinylated, and the biotinylated form was shown to be preferentially internalized by rat pheochromocytoma cells as compared to non-biotinylated hemoglobin.

Example 3 - SOYBEAN CELL UPTAKE OF BOVINE SERUM ALBUMIN:

Soybean cell suspension cultures of Glycine max Merr Var Kent were maintained by transferring cells to fresh W-38 growth medium every 7 days.

To 20 ml of a suspension culture of soybean cells was added 10 g of either fluorescein-labeled (control) or fluorescein and biotin labeled bovine serum albumin. The cells were allowed to incubate for up to 6 hours. At

varying time intervals 1 ml of the cell suspension was filtered to remove the growth medium, washed with 50 ml fresh growth medium, and resuspended in 20 ml of the same medium. The cell suspension was then viewed with a fluorescent microscope to determine whether cellular internalization of the labeled bovine serum albumin had occurred. Cellular internalization was indicated only for biotinylated bovine serum albumin.

<u>Example 4</u> - SOYBEAN CELL UPTAKE OF INSULIN:

Following the same general procedure set forth in Example 3 insulin was biotinylated, and the biotinylated form of insulin was shown to be preferentially internalized by soybean cells as compared to non-biotinylated insulin.

<u>Example 5</u> - SOYBEAN CELL UPTAKE OF HEMOGLOBIN:

Following the same general procedure set forth in Example 3 hemoglobin was biotinylated, and the biotinylated form of hemoglobin was shown to be preferentially internalized by soybean cells as compared to non-biotinylated hemoglobin.

<u>Example 6</u> - CARROT CELL UPTAKE OF BOVINE SERUM ALBUMIN:

Carrot cells of wild type origin were established and maintained in MS growth medium supplemented with 0.1 mg/L 2,4-dichlorophenoxyacetic acid. Bovine serum albumin was labeled with fluorescein alone as a control or with fluorescein and biotin following the procedures detailed in Example 3. The carrot cells were then incubated in the presence of the respective labeled bovine serum albumin for 7 hours. All other conditions were the same as those described in Example 3 above. Cellular internalization was found only in those cells contacted with biotin labeled bovine serum albumin.

Example 7 - CARROT CELL UPTAKE OF INSULIN:

Following the same general procedure set forth in Example 6 insulin was biotinylated, and the biotinylated form was shown to be preferentially internalized by carrot cells as compared to non-biotinylated insulin.

Example 8 - CARROT CELL UPTAKE OF HEMOGLOBIN:

Following the same general procedure set forth in Example 6 hemoglobin was biotinylated, and the biotinylated form was shown to be preferentially internalized by carrot cells as compared to non-biotinylated hemoglobin.

Example 9 - SOYBEAN CELL DEGRADATION OF HEMOGLOBIN:

To determine whether hemoglobin was rapidly degraded following cellular internalization by transmembrane transport, soybean cells were allowed to internalize and metabolize biotinylated hemoglobin for a period of 8 hours under conditions described in Example 5, after which the soybean cells were rapidly homogenized in a sodium dodecyl sulfate solution to disaggregate and denature all protein material. The solubilized polypeptides were separated according to molecular weight by polyacrylamide gel electrophoresis and then electroblotted onto nitrocellulose paper. The positions of the biotin-labeled peptides were then visualized on the nitrocellulose blot by staining with horseradish peroxidase-linked avidin and the colored substrate, p-chloronaphthol. All of the biotin-linked material was found to migrate with an apparent molecular weight of 16,000 daltons, about equal to the molecular weight of the parent globin chains of hemoglobin, indicating no breakdown of the parent globin chains had occurred during the 8 hour incubation period.

Example 10 - *IN VIVO* DELIVERY TO MICE OF SOYBEAN TRYPSIN INHIBITOR:

Soybean trypsin inhibitor (SBTI) (6 mg) was labeled with radioactive ^{125}I using 8 iodobeads (Bio Rad) in 1 mL buffer which was then dialyzed to remove unreacted ^{125}I. After dividing into two equal fractions, one fraction was biotinylated with N-hydroxysuccinimidyl biotin and the other fraction was left as an unmodified control. Mice (25 g) were then injected with either the biotinylated SBTI or the control SBTI by insertion of a hypodermic syringe containing a 25 gauge needle into the tail vein of the mouse. After 15 minutes, each mouse was sacrificed and then perfused with heparin-containing isotonic saline via the direct cardiac influx and efflux method. When the various tissues appeared to be blood-free, the perfusion was terminated and each tissue/organ was removed, weighed, and counted for ^{125}I-SBTI in a gamma counter. Although some radioactivity was detected in the mice treated with non-biotinylated ^{125}I-SBTI, between 4 and 100 times more ^{125}I-SBTI was found in the mice treated with biotinylated SBTI, indicating successful *in vivo* delivery to murine cellular tissue.

	Counts per minute/gram wet weight	
Tissue	Control SBTI	Biotin SBTI
Liver	535	1967
Lung	107	2941
Kidney	5152	8697
Intestine	0	700
Muscle	0	1065
Heart	0	739
Brain	0	267

Example 11 - SOYBEAN CELL UPTAKE OF SALMON SPERM DNA:

Protein free salmon-sperm DNA, either in a highly polymerized form (≥ 50,000 base pair length) or in a sheared form (≤ 500 base pair length), was transaminated at the cytosine residues. The transaminated DNA (1 mg) was labeled with fluorescein via the addition of 0.5 mg of fluorescein isothiocyanate (FITC) in dimethylsulfoxide (DMSO). The resulting reaction mixture was divided into two portions and the labeling reaction was quenched in one portion by addition of 10 L of ethanolamine. This quenched portion served as the non-biotinylated control. The remaining DNA was then covalently labeled with biotin via reaction with 0.5 mg of N-hydroxysuccinimidyl biotin in DMSO. After purification, the two derivatives (1 g/ml) were separately incubated with soybean suspension culture cells at room temperature for 6 hours and then the cells were washed with 50 ml fresh growth medium and observed by fluorescence microscopy. Only the biotinylated DNA entered the soybean cells.

Example 12 - E. COLI TRANSFORMATION AND EXPRESSION OF AMPICILLIN RESISTANT GENE:

Plasmid DNA (pUC8) was biotinylated via nick translation in the presence of biotin-14-dATP using a commercially available nick translation kit (Bethesda Research Laboratories). The biotinylated DNA and unmodified DNA (1 g) were added to E. coli strain Cu 1230 that had been made competent by treatment with $MgCl_2$ and $CaCl_2$ following the method of Maniatis et al., Molecular Cloning: A Laboratory Manual, pp. 250-251, Cold Spring Harbor Press (1987). After transformation, the successful transformants were selected by plating cells on LB media which contained 50 g/ml ampicillin and then incubated overnight at 37°C. Colonies which survived the ampicillin were counted and the transformation efficiency was

determined. The number of surviving E. coli colonies was at least 100-fold greater in E. coli transformed with the biotinylated plasmids.

Example 13 - BLOCKADE OF DELIVERY OF BIOTINYLATED PROTEINS INTO SOYBEAN CELLS BY COMPETITION WITH UNLIGATED BIOTIN:

Insulin, ribonuclease (RNase) and bovine serum albumin (BAS) were individually biotinylated following the same general procedure set forth in Example 1 above. A sample of each of the biotinylated proteins and an unmodified sample of the same protein (control protein) were radioiodinated according to the following protocol. To 1 mL of a 200 mM phosphate buffer, pH 7.0, containing 3 iodobeads (Pierce Chemical Co.) was added 0.2 mCi [^{125}I]-NaI (carrier-free in 1 n NaOH, Amersham) and the mixture was allowed to incubate for 5 minutes to liberate the active iodine species, according to the supplier's instructions. After activation, 1 mg of desired biotinylated or control protein was added in 0.5 mL of iodination buffer. The iodination was allowed to proceed with stirring for 20 minutes. After the iodination was complete, the product was isolated via gel filtration on a Biogel PH-10 column. Typical iodinations of ribonuclease A (Sigma Chemical Co.) yielded a product emitting 2 x 10^5 cpm/ g.

Uptake of ^{125}I-labeled proteins by soybean suspension culture cells in the early exponential growth phase was then assayed as follows. To each culture was added sufficient ^{125}I-labeled macromolecule to achieve a final concentration of 10 g/mL, and the suspension was incubated at 23° for the desired time. After the desired incubation period, the cells were washed for 5 minutes in growth media rebuffered to pH 8 with 15 mM glycylglycine to remove surface bound ligand. The cell suspension was then filtered, washed with 200 volumes growth media, and placed in counting vials.

Uptake of biotin-conjugated RNase was rapid, reaching 6 x 10^6 molecules internalized per cell in the first 3 hours. In contrast, unmodified RNase was not internalized, demonstrating the importance of the biotin adduct. To further confirm the role of biotin in mediating the delivery of RNase, the cell suspension was treated with 1 mM free biotin directly prior to addition of the biotin-derivatized RNase. Free biotin competitively blocked delivery of the conjugated protein into the soybean cells. Therefore, it can be concluded that the internalization process involves recognition of biotin by a limited number of receptors on the plant cell surface.

Similar studies with biotin-labeled BSA and insulin yielded virtually identical results.

Example 14 - PARTIAL PURIFICATION OF BOVINE SERUM ALBUMIN FOLLOWING ITS INTERNALIZATION BY CULTURED SOYBEAN CELLS:

Radiolabeled, biotinylated bovine serum albumin was allowed to bind and enter cultured soybean cells following the same general procedure set forth in Example 13, after which the cells were thoroughly washed, homogenized and extracted to remove cytoplasmic soluble proteins. This cytoplasmic protein extract was separated using standard chromatographic techniques on a Sephadex G-25 gel filtration column to determine whether any small molecular weight fragments might be generated during the co-delivery process. Comparison of the elution profile of the ^{125}I-labeled material isolated from the cell extract with the profile of unmodified ^{125}I-serum albumin showed that the majority of the internalized protein remained intact throughout the 2 hour duration of the internalization study.

<u>Example 15</u> - RESTORATION OF GROWTH IN CULTURED CELLS DEFICIENT IN HYPOXANTHINE-GUANINE PHOSPHORIBOSYL TRANSFERASE (HGPRT) UPON ADDITION OF BIOTINYLATED - HGPRT.

Cells deficient in HGPRT (i.e., the defect in Lesch-Nyhan Syndrome) are able to grow only in a cellular growth medium containing hypoxanthine, aminopterin and thymidine, (HAT), supplemented with purines. However, these same cells were found to grow normally in HAT medium after internalization of biotin-linked HGPRT via the biotin-mediated endocytosis pathway. HGPRT was biotinylated in the presence of hypoxanthine and phosphoribosyl pyrophosphate (to protect the active site) with N-hydroxysuccinimido biotin. The crosslinked enzyme retained 55% of the original activity and SDS PAGE analysis followed by transblotting and avidin-peroxidase binding indicated that a 1-4 biotins were attached per molecules of HGPRT. HGPRT deficient fibroblasts (GM 00152) incubated with biotinylated HGPRT (4.6 x 10^4 units/cell) grew at a rate comparable to cells supplemented with purines for at least 24 hours. Appropriate control incubations did not grow on HAT medium supplemented with HGPRT, biotin, phosphoribosyl, and inosine monophosphate.

<u>Example 16</u> - TRANSFORMATION OF CULTURED SOYBEAN CELLS WITH A KANAMYCIN RESISTANCE GENE USING THE BIOTIN DELIVERY SYSTEM:

The expression vector pGA642-643 containing a bacterial kanamycin resistance gene was nicked with <u>Eco</u>R1 and the sticky ends were filled in using biotinylated ATP and a T4 polymerase-based nick translation kit following the general procedure set forth in Example 12. Identical control plasmids were left unmodified. Then, to 40 ml of a soybean cell suspension was added either the biotinylated plasmid or the control (nonbiotinylated) plasmid. After incubation for 10 hours, the cells from each flask were transferred to fresh growth medium containing 100 g/ml

kanamycin and allowed to proliferate under normal conditions. Each flask was also transferred to fresh medium containing 100 g/ml kanamycin every 3 days. By day 10, the flask treated with the biotinylated plasmid had increased 6-fold in cell mass, while the flask treated with the control plasmid exhibited no measurable growth.

Example 17 - USE OF FOLIC ACID CONJUGATION TO DELIVER RIBONUCLEASE INTO CULTURED HUMAN CELLS:

Activated folic acid was prepared by dissolving 1 mg of folic acid and 3.8 equivalents of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in 0.5 ml of dimethylsulfoxide (DMSO). The solution was allowed to set for 2.5 hours. A sample of folate-labeled bovine ribonuclease was prepared by treating the ribonuclease with 34-fold molar excess of EDC-activated folate. The resulting derivatized RNase contained 12-14 covalently bound folates per protein molecule. A second sample of the ribonuclease was left unmodified to serve as a control. The folate-labeled sample and the control sample were radioiodinated following the same general procedure set forth in Example 13. Following exhaustive dialysis, the two ^{125}I-labeled samples were added to KB cells (a human nasopharyngeal cell line) and examined for uptake of ^{125}I-RNase after 30 minutes. No protein uptake was seen for RNase control samples, while 10^7 molecules per cell were internalized by the RNase labeled with folate (RNase-Folate). To confirm that the uptake was indeed folate-mediated, the KB cells were treated with either control RNase or folate-labeled RNase in the presence of a 100-fold molar excess of unligated folate (100X). The control RNase again displayed no internalization; uptake of the RNase-Folate conjugate was reduced 7-fold by competitive inhibition. Similar studies yielded corresponding results using human HeLa cells.

Example 18 - USE OF FOLIC ACID CONJUGATION TO DELIVER SOYBEAN TRYPSIN INHIBITOR (SBTI) INTO CULTURED HUMAN CELLS:

Experiments following the general procedure set forth in Example 17, with soybean trypsin inhibitor being substituted for ribonuclease, were conducted with virtually identical results. Folate ligation was again demonstrated to be essential for uptake of SBTI by KB cells.

Example 19 - VISUALIZATION OF RIBONUCLEASE ENDOCYTOSIS BY KB CELLS USING A CONFOCAL MICROSCOPE:

Bovine ribonuclease (RNase) was labeled with fluorescein isothiocyanate following the same general procedure set forth in Example 1 and then further labeled with folate following the same general procedure set forth in Example 17. RNase labeled only with fluoroscein was used as a control. Following extensive dialysis against growth medium, the control and folate-labeled RNase samples were added to separate cultures of KB cells. After 60 minute incubation, the cells were thoroughly washed and examined for uptake. Only the folate-labeled samples displayed any internal fluorescence when viewed with laser excitation under the confocal microscope (Bio Rad). Furthermore, using the confocal's capability of focusing on a single horizontal plane in each cultured cell, it was readily evident that vesicles filled with the fluorescent-labeled, folate-bound ribonuclease were forming on all regions of the cell surface, pinching off via endocytosis into the interior, and entering the cytoplasm. The vesicles, measuring 0.8 to 1.0 m across, were easily large enough to accommodate large biomolecules such as proteins and DNA plasmids.

Example 20 - UPTAKE OF RIBONUCLEASE IN COMPLEX WITH FOLATE BY WHITE BLOOD CELLS:

Fluorescein-labeled RNase was either conjugated to folate or left unmodified (control) following the same general procedure set forth in Example 19. The folate-conjugated and control samples were then added to freshly drawn whole human blood, incubated at 37°C for 2 hours and then washed thoroughly and examined under the fluorescence microscope. Cells bearing folate receptors that were brought into contact with the RNase/folate/fluorescein complex were found to fluoresce. None of the control cells exhibited fluorescence.

Example 21 - *IN VIVO* DELIVERY OF RIBONUCLEASE THROUGHOUT TISSUES OF LIVE MICE FOLLOWING INTRAVENOUS INJECTION:

Ribonuclease was labeled with ^{125}I following the same general procedure set forth in Example 13 and then further conjugated with folate or left unmodified to serve as a control, following the general procedure set forth in Example 17. Live mice were injected with either the folate-conjugated or control sample by inserting a 27 gauge needle into the tail vein of the mice and injecting 0.2 ml of the appropriate sample dissolved in physiological saline. After 1 hour, the mice were anesthetized, perfused with saline and dissected to determine the specific radioactivity of each organ, following the general procedure set forth in Example 10. Uptake was determined by relative comparison of specific radioactivity of the various tissues examined (units compared were counts per minute/gram of tissue divided by the specific activity of a blood sample drawn 3 minutes after injection, i.e., in order to normalize for any variability in the amount injected). Folate conjugation provided greatly enhanced uptake by the liver and lung, while the kidney, an organ

responsible for clearance of unwanted proteins, was enriched in unmodified RNase.

Similar results were obtained when the mice were allowed to live for 18 hours post-injection, with preferential uptake of folate-conjugated RNase also being noted in the intestine, heart, muscle and brain.

<u>Example 22</u> - *IN VIVO* DELIVERY OF RIBONUCLEASE THROUGHOUT TISSUES OF LIVE MICE FOLLOWING INTRAPERITONEAL INJECTION:

Folate-derivatized and control RNase (^{125}I-labeled) were prepared as described in Example 21 and injected into the peritoneal cavity of 30g mice using a 27 gauge needle and syringe. After 17 hours, the mice were anesthetized, perfused, and dissected to remove various body tissues. Each tissue was then weighted and counted for radioactivity. The specific uptake of both the control and folate-conjugated RNase were compared following the general procedure set forth in Example 21. As compared to intravenous administration, intraperitoneal injection resulted in enhanced delivery of the folate-derivatized RNase to all tissues except the kidney. Transmembrane delivery across the blood/brain barrier was demonstrated by the brain tissue's preferential uptake of the folate-labeled protein. Similar results were obtained in two other repetitions of the foregoing procedure.

Example 23 - REVERSION OF src-TRANSFORMED FIBROBLASTS TO DIFFERENTIATED STATE UPON TREATMENT WITH ANTI-SENSE DNA CONJUGATED TO FOLATE:

A pentadecameric oligonucleotide DNA probe of the formula

$$5'\text{-CTTGCTACTCCCAT-O-}\underset{\underset{-\text{O}}{|}}{\overset{\overset{\text{O}}{|}}{\text{P}}}\text{-O-}(CH_2)_8\text{-}NH_3{}^{+}$$

complementary to a sequence spanning the initiation codon of the Rous sarcoma src oncogene and containing a free 3′ amino group was derivatized with folate using carbodiimide chemistry. A second sample was left unmodified as a control. Both samples were dissolved in phosphate buffered saline and introduced into culture dishes containing fibroblasts transformed by the Rous sarcoma virus (XC cells) at a final oligonucleotide concentration of 8×10^{-6} M. After 24 hours, the cultured cells were viewed under a microscope. Results showed that 40% of the cells treated with the folate/antisense oligonucleotide complex had reverted to the normal fibroblast-like morphology, while only 10% of the controls displayed the same nontransformed phenotype. The remaining cells in both culture dishes retained their highly rounded shape characteristic of the neoplastic state.

Example 24 - RIBOFLAVIN ENHANCED UPTAKE OF MACROMOLECULES:

Methods of conjugating riboflavin to macromolecules

Three methods have been used to covalently link riboflavin to a proteins. All 3 methods have been shown to enable the delivery of attached macromolecules as diverse as BSA (Mr - 68,000), momordin (Mr - 22,000) and

ribonuclease (Mr - 13,700) nondestructively into living cells (vide infra). The first method involves oxidation of the side chain of riboflavin first with periodate and then further with permanganate, followed by carbodiimide mediated coupling of the generated carboxylate to N-hydroxysuccinimide (NHS). The NHS ester of the modified riboflavin can then react with any primary or secondary amine, such as the lysine side chains present on the surface of BSA.

The NHS-riboflavin can be reacted with cysteamin to generate a riboflavin derivative with a free sulfhydryl at the end of a short spacer. This spacer is then lengthened by reaction of the sulfhydryl with maleinidylbenzoyl NHS. The resulting NHS derivative of riboflavin is similarly reactive toward primary amines, only the vitamin is separated from the conjugated protein by a 12 atom spacer.

A different reaction scheme can be used to conjugated riboflavin to a protein or other macromolecule as follows. Unmodified riboflavin is first reacted with succinic anhydride to extend the vitamin at the site of the primary hydroxyl. The free carboxylate is then activated as described above with NHS in the presence of a carbodiimide. The resulting derivative can link riboflavin to any amine-containing molecule by a 5 atom spacer.

Quantitative analysis of ribonuclease-riboflavin uptake by BHK cells.

Ribonuclease was labeled with ^{125}I as described by Leamon and Low (1991) Proc. Natl. Sci. USA 88, 5572-5576, and then either further derivatized with riboflavin or left unmodified. The samples (10 $\mu g/ml$) were then added to 50% confluent monolayers of BHK cells and incubated for various times. At various times as shown on the abscissa of Fig. 4, the cells were washed 5X in saline, and removed and

counted in a gamma counter. The ribonuclease sample derivatized with 6 riboflavins per protein (RNase A-Rf (6)) resulted in the highest rate of uptake followed by the sample conjugated to just one riboflavin (RNase A-Rf (1)). The two samples lacking riboflavin (RNase-control (6) and RNase-control (1)) endocytosed little or no ^{125}I-ribonuclease. (see Fig. 4)

Analysis of riboflavin-mediated protein uptake by various tissues in live rats.

^{125}I-BSA(40 μg) derivatized further with riboflavin (2.3 moles/mole BSA) or left unmodified was injected into the tail vein of Wistar white female rats and allowed to circulate 1 hr. The rats were then anesthetized, perfused through the left ventricle of the heart with phosphate buffered saline containing 10 U/ml heparin until the effluent from the right ventricle appeared clear and the organs appeared pale, and the rates were then dissected. The specific radioactivities of each organ were then determined [see Fig. 5, the solid bars represent the BSA content of tissues from rats treated with the ^{125}I-BSA-riboflavin conjugated samples, while the open bars represent the controls (^{125}I-BSA)]. Clearly, conjugation with riboflavin enhances tissue retention/uptake manyfold in live rats.

Example 25 - THIAMIN ENHANCED UPTAKE OF MACROMOLECULES:

Protocol for Thiamin Coupling:

Thiamin was dried at 95°C for 4 hours. To 200 mg dry thiamin 320 μl thionyl chloride was added with stirring in an ice bath for 10 minutes and then 40 μl pyridine was added in same way. The mixture was stirred at 0°C for 10 minutes and then was moved to a 50°C oil bath and reacted for an additional 60 minutes. White thiamin was washed

with ether 4 times and then dried by aspiration for a half hour. The dry product was stored at 4°C.

10 mg of activated thiamin in small aliquots was added to 1 ml of a 10 mg/ml Bovine Serum Albumin/PBS solution. The pH of the mixture was maintained at 8. After stirring for 10 minutes the solution changed to deep green color. The mixture was centrifuged at 5000 g. for 20 minutes at 4°C. The liquid phase containing the labeled BSA was saved for purification.

The labeled BSA was purified by G-75-120 gel filtration chromatography in pH 7.4 PBS two times. The fast moving green band was the BSA-Thiamin. The amount of thiamin attached to BSA was estimated by oxidizing the conjugated thiamin to thiochrome and measuring the level of fluorescence at excitation 365 nm and emission 445 nm. The oxidation of the conjugated thiamin was preformed as follows: 20μl BSA-thiamin solution was diluted to 2 ml with dH_2O. 20μl 2% K_3Fe (CN) 6 and 20 μl 1M NaOH were added to the above solution. The mixture was vortexed for 10 seconds, allowed to stand for 10 minutes, then evaluated for the level of fluorescence. BSA concentration was estimated by the Bicinchoninic Acid (BCA) method.

0.5 mg fluorescein isothiocyanate/100 μl dimethyl formamide was added to 1 ml of a 2mg BSA-thiamin/ml solution. The pH of the mixture was maintained at 8 while the reaction was stirred at room temperature for 3 hours. FITC-BSA-thiamin was separated from free FITC by two rounds of G-75-120 gel filtration chromatography. The average number of FITC molecules conjugated to BSA was estimated by measuring the absorbance at 495 nm.

<u>Cell Culture and Uptake Protocol</u>:

KB Cells were cultured in thiamin deficient Eagle's minimum essential medium consisting of 10% fetal bovine serum (heat denatured), 100 Units/ml penicillin,

100μg/ml streptomycin, 2μg/ml amphotericin B and 2 mM glutamine for more than two passages. KB Cells were transferred to 6 cm culture dishes and cultured at 37°C for two days (until about 90% confluent). The medium in each dish was replaced with 2 ml new thiamin deficient MEM and the cells were incubated with BSA-thiamin, as desired.

Treated cells were cultured at 37°C for 3 hours. The culture dishes were then washed with PBS four times and the cells of each dish were scraped and collected into a 1 ml centrifuge tube. The cells in the tubes were washed with PBS 4 times, and then spun down and lysed with 1 ml 1% Triton-X 100.

The lysis solutions were assayed for fluorescence at excitation 495 nm and emission 520 nm, and the protein content was estimated by the Bicinchoninic Acid (BCA) method. From the fluorescence one can calculate the total number of BSA molecules taken up by the KB cells in each dish, and from the protein concentration one can calculate the cell number in each dish. The ratio of the two measurements yields the number of BSA-thiamin molecules internalized per cell.

Example 26 - THIAMIN & RIBOFLAVIN UPTAKE BY A549 CELLS

Observation of thiamin & riboflavin mediated protein uptake by fluorescence microscopy

Bovine serum albumin was labeled, with fluorescein isothiocyanate (FITC) and optionally further complexed with either thiamin or riboflavin. A549 cells were incubated with FITC-BSA, FITC-BSA-thiamin or FITC-BSA-riboflavin in accordance with the procedure described above. The accumulated data is represented by Figure 6. As the data indicates, the uptake of BSA was enhanced when BSA is conjugated with thiamin or riboflavin as compared to BSA conjugated to FITC alone. Applicants have also discovered

that serum contains a binding protein that competes with the cellular receptors for thiamin. Removal of serum prior to incubating the cells with thiamin conjugated BSA, further enhances the uptake of the conjugated BSA complex.

Time Dependent of Uptake of BSA and BSA-Thiamin by KB Cells

The time-dependent uptake of thiamin-BSA-FITC conjugates by KB cells has been measured. (*See* Figure 7). Solid circles represent the FITC-BSA conjugate, and the solid squares and solid triangles represent thiamin-BSA-FITC conjugates, wherein the solid squares represent an average of 1.8 molecules of thiamin per BSA molecule and the solid triangles represent 3.9 molecules of thiamin per BSA molecule. As the data indicates both thiamin-BSA-FITC conjugates are taken up to a much greater extent than the BSA-FITC conjugates.

Example 27 - PREPARATION AND PURIFICATION OF FOLATE-DEFEROXAMINE CONJUGATES:

Materials. Folic acid, deferoxamine (DF) mesylate, and DEAE-trisacryl anion-exchange resin were purchased from Sigma (St. Louis, MO). Bicinchoninic acid (BCA) protein assay kit was obtained from Pierce (Rockford, IL). Acetonitrile (HPLC grade) and dicyclohexylcarbodiimide (DCC) were purchased from Aldrich (Milwaukee, WI). Gallium-67 chloride was purchased from Mallinckrodt Medical, Inc. (St. Louis, MO). Tissue culture products were obtained from GIBCO (Grand Island, NY), and cultured cells were received as a gift from the Purdue Cancer Center (West Lafayette, IN).

100 mg DF mesylate was dissolved in 3 mL dimethylsulfoxide containing 200 μL pyridine. A 10-fold excess of folic acid (672 mg) was dissolved in 15 mL warm (~40°C) dimethylsulfoxide and 5 molar equivalents of DCC (157 mg) were then added. The reaction mixture was stirred

at 40°C in the dark, during which ninhydrin assay and thin layer chromatography were used to follow the reaction process. After the coupling was complete, the DF-folate conjugate and excess folic acid were precipitated with 200 mL cold acetone and pelleted by centrifugation. The pellet was washed once with cold acetone, dried under vacuum and then redissolved in 5 mL deionized water. The pH of the solution was adjusted to 8.0 to facilitate dissolution of the solid.

The crude product contained a mixture of folate-deferoxamine conjugates, wherein the folate is linked to DF via its α-carboxyl or γ-carboxyl group as well as unreacted folic acid. The two isomers of DF-folate were isolated and purified on a weak anion-exchange column. Briefly, the product mixture was loaded onto a 1.5 cm X 15 cm DEAE-trisacryl column pre-equilibrated in 10 mM NH_4HCO_3 buffer (pH 7.9). The column was washed with 50 mL 10 mM NH_4HCO_3 and then eluted with a 500 mL gradient of 80-180 mM NH_4HCO_3 followed by 150 mL 500 mM NH_4HCO_3. Three folate-containing peaks were obtained as detected by UV absorbance at 363 nm. Each peak was collected, lyophilized, and redissolved in deionized water. The purity of each component was confirmed by reverse-phase high pressure liquid chromatography (HPLC) with a 10 mm X 250 mm Licrosorb RP-18 column (Altech, Deerfield, IL), and evaluation of the conjugates' molecular weights was determined by fast-atom bombardment mass spectroscopy (FAB-MS). The characteristic pKa values of the two DF-folate isomers were obtained by titration on a pH/ion analyzer (Corning, Corning, NY).

The first two peaks were identified as DF-folate conjugates, both giving a molecular weight of 984.0 in their FAB-MS spectra indicating the expected 1:1 ratio between folic acid and DF. The third peak showed the same molecular weight as free folic acid. Since the two isoforms of DF-folate conjugate retain either a free γ-

carboxyl or free α-carboxyl, they can be distinguished from each other and from unreacted folic acid by their characteristic pKa values, which were determined by titration. The DF-folate(α) conjugate (pKa=2.5, constituting ~20% of total DF-folate) eluted in 140-260 mL fractions (peak 1), the DF-folate(γ) conjugate (pKa=4.5, constituting ~80% of total DF-folate) eluted in 340 mL to 420 mL fractions (peak 2), and the free folic acid (pK_{a1}=2.5, pK_{a2}=4.5) eluted between 580 mL to 680 mL (peak 3).

<u>Example 28</u> - PREPARATION OF ^{67}GA-RADIOTRACERS:

^{67}Ga-deferoxamine-folate conjugate, ^{67}Ga-citrate, and ^{67}Ga-deferoxamine were prepared from no-carrier-added ^{67}Ga-gallium(III) chloride (Mallinckrodt Medical, Inc. St. Louis, MO). The ^{67}Ga-deferoxamine-folate conjugate was prepared as follows: A dilute HCl solution of $^{67}Ga^{3+}$ was evaporated to dryness with heating under a stream of N_2 and the tracer reconstituted in 300 μL ethanol containing 0.002% acetylacetone (acac). The ethanolic $^{67}Ga(acac)_3$ solution (3.2 mCi) was diluted with an equal volume of TRIS-buffered saline (pH 7.4) followed by addition of 2.25 X 10^{-6} mole of aqueous DF-folate(γ) conjugate. Labeling was complete after standing at room temperature for 18-24 hours.

^{67}Ga(III)-citrate was prepared by evaporating a ^{67}Ga-chloride solution to dryness and reconstituting with 0.1mL of 3% sodium citrate (pH 7.4). A portion of the resulting ^{67}Ga-citrate solution (50 μL) was mixed with 0.1 mg deferoxamine to obtain ^{67}Ga-deferoxamine (^{67}Ga-DF).

The radiochemical purity of the ^{67}Ga-tracers was determined by thin layer chromatography on C_{18} reverse phase silica gel plates eluted with methanol and in all cases was found to exceed 98%. The radiochromatograms were evaluated

using a Berthold (Wildbad, Germany) Tracemaster 20 Automatic TLC Linear Analyzer. Rf values of 0.93; 0.0; 0.1; and 0.74 were obtained for ^{67}Ga-DF-folate(γ); ^{67}Ga(acac)$_3$; ^{67}Ga-DF; and ^{67}Ga-citrate, respectfully. All experiments employing the ^{67}Ga-DF-folate(γ) tracer were performed within 1-3 days of preparation.

Example 29 - DETERMINATION OF THE AFFINITIES OF THE TWO DF-FOLATE ISOMERS FOR CELL SURFACE FOLATE RECEPTORS:

Cell Culture. KB cells, a human nasopharyngeal epidermal carcinoma cell line that greatly overexpresses the folate binding protein, were cultured continuously as a monolayer at 37°C in a humidified atmosphere containing 5% CO_2 in folate-deficient modified Eagle's medium (FDMEM) (a folate-free modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum as the only source of folate) containing penicillin (50 units/mL), streptomycin (50 μg/mL), and 2 mM L-glutamine. The final folate concentration in the complete FDMEM is the physiological range (~2nM). 48 h prior to each experiment, the cells were transferred to 35 mm culture dishes at 5 X 10^5 cells per dish and grown to ~80% confluent.

The affinity of the DF-folate(α) and DF-folate(γ) conjugates for the KB cell folate-binding protein was evaluated in a competitive binding assay using [^{3}H]folic acid as the receptor ligand. Briefly, 100 pmoles of [^{3}H] folic acid and 100 pmoles of either DF-folate(α) or DF-folate(γ) dissolved in phosphate-buffered saline (PBS) were added to KB cells grown ~80% confluence in 1 mL FDMEM in 35 mm culture dishes. Following a 30 min incubation at 4°C, the cells were washed 3 times with cold PBS. Cell-associated [^{3}H] folic acid was then determined by liquid scintillation counting, and the cellular protein content was evaluated by the BCA protein assay.

Assuming a cellular protein content of ~2 X 10^{-7}g, 4.85 x 10^{-6} folate receptors were occupied with the radiolabeled ligands on each cell. A 50% decrease in bound [^{3}H]folic acid was observed in the presence of an equimolar amount of the DF-folate(γ) conjugate, while the DF-folate(α) conjugate display no ability to compete with the radiolabeled vitamin. The competition by DF-folate(γ) was similar to that of unlabeled folic acid, indicating that covalent conjugation of DF to the γ-carboxyl of folic acid does not compromise the latter's high affinity for the membrane-associated folate binding protein.

<u>Example 30</u> - UPTAKE OF ^{67}GA-DF-FOLATE COMPLEX BY CULTURED KB CELLS:

Because folate and its conjugates bind to cell surface receptors at 4°C, but are capable of endocytosis only at higher temperatures, it is possible to separately evaluate the kinetics of binding the internalization of folate by measuring the rates of folate conjugate uptake at the two temperatures. Half maximal binding of ^{67}Ga-DF-folate(γ) was achieved in ~3 min at 4°C, suggesting rapid association of the conjugate with unoccupied receptors. By the end of the 30 min incubation, binding approached to saturation with ~18% of the initial radioactivity found associated with a cell surface.

Incubation at 37°C, a temperature which permits both binding and endocytosis, yielded similar kinetic results, however, maximal uptake reached 32% of the total conjugate added. Presumably, the difference in magnitude of the two cellular uptake curves reflects the ability of the folate receptor to internalize the conjugate and then recycle to the cell surface in its unoccupied form. As controls, ^{67}Ga-DF lacking the folate group did not show any significant uptake by the KB cells, nor did the ^{67}Ga-DF-folate(α) complex. This latter result is consistent with the inability of the α-conjugate to compete with free

folate for the cell surface receptor. When ^{67}Ga-citrate was added to the culture medium and incubated at 37°C for 30 min, cell-associated ^{67}Ga radioactivity was 106-fold lower than observed with ^{67}Ga-DF-folate(γ).

To verify the involvement of a cell surface folate receptor in mediating the uptake of ^{67}Ga-DF-folate(γ), binding and internalization of the complex were evaluated as a function of the complex's concentration. Cellular uptake of ^{67}Ga-DF-folate(γ) was concentration dependent at both 4°C and 37°C, saturating at levels of 20% and 35% of the total radioactivity added, respectively. Analysis of competition between ^{67}Ga-DF-folate(γ) and unlabeled folic acid further demonstrated that cellular uptake was folate receptor-mediated by specific binding, since only 0.5% of initial cellular uptake was retained in the presence of 100-fold molar excess of free folate. A 50% decrease in uptake was again observed when equimolar amounts of ^{67}Ga-DF-folate(γ)/DF-folate(γ) and unlabeled folic acid were mixed and then added to the cell culture, suggesting that the affinity of the radiolabeled conjugate for the membrane-associated folate receptor is comparable to that of the metal-free conjugate. In aggregate, these results suggest that ^{67}Ga-DF-folate(γ) associates with cell folate receptors in much the same manner as free folic acid.

Example 31 - IMAGING WITH A ^{67}GA-DEFEROXAMINE-FOLATE CONJUGATE:

To test the viability of the FBP-receptor as a pathway for achieving tumor-cell-selective uptake of metal-labeled radiopharmaceuticals *in vivo*, ~180 μCi of ^{67}Ga-deferoxamine-folate conjugate was administered intravenously to two tumor-bearing athymic mice *via* the femoral vein nine days after subcutaneous injection of 4 X 10^6 human KB cells. At approximately 45 hours post-

injection, gamma images were obtained, the animals were sacrificed and dissected, and the tissue distribution of tracer was quantified by gamma counting (after storage of the weighted tissue samples for decay to a suitable ^{67}Ga count rate). The tumors from the two animals were found to have masses of 29.6 and 8 mg, while the total body mass of the two animals was 19.3 and 22.6 grams, respectively. Despite the small size and sub-optimal positioning of these tumors relative to the kidneys, the 29.6 mg tumor was readily detected by gamma scintigraphy. At sacrifice the 29.6 mg tumor was found to contain 3.3% of the injected dose per gram of tumor, while the 8 mg tumor contained 2.8% of the injected dose per gram of tumor. The tumor to blood ratio was 1500 and 1185 and the tumor to muscle ratio was 170 and 288 for each respective mouse.

<u>Example 32</u> - IMAGING AND RADIOTRACER BIODISTRIBUTION STUDIES WITH A ^{67}GA-DEFEROXAMINE-FOLATE CONJUGATE:

To better define the ability of ^{67}Ga-deferoxamine-folate to target tumor cells *in vivo* and to confirm the role of the FBP receptor in determining conjugate tumor uptake, an additional study was conducted using 17 athymic mice. Male athymic mice (Nu/Nu; 21-28 days old) were housed under aseptic conditions, and fed a folate-deficient diet from the time of receipt, unless otherwise indicated. Folate-deficient rodent chow was obtained from ICN Biochemicals and autoclaved prior to use. Animals were anesthetized with ketamine (40 mg/kp, i.p.) and xylazine (4 mg/kg, i.p.) for radiopharmaceutical injection, for gamma imaging studies, and again prior to sacrifice. Syringes used for radiotracer injections were weighted on an analytical balance before and after injection to quantitate the dose received by each animal.

A Capintec CRC12R Radionuclide Dose Calibrator was used for appropriate assays of ^{67}Ga; while samples requiring

precise quantitation of ^{67}Ga were counted in a Packard 5500 Automatic Gamma Scintillation Counter with 3-inch large-bore NAI(Tl) crystal. Gamma images of intact animals were obtained using a Searle 37GP gamma scintillation camera fitted with a 300 keV parallel hole collimator and linked to a Siemens MicroDELTA computer.

Fifteen days after subcutaneous injection of 4 X 10^6 human KB cells into the shoulder of the mice, each animal received 125-150 μCi of either ^{67}Ga-DF-folate (11 animals; Groups 1-4), ^{67}Ga-DF (3 animals; Group 5), or ^{67}Ga-citrate (3 animals; Group 6) *via* intravenous injection into the femoral vein. Injection volumes were approximately 130 μL of 10% ethanol in saline per animal. All animals except two were maintained on folate-deficient diet for 3 weeks prior to radiotracer administration; the remaining two animals were maintained on normal rodent chow and included in the animals that received ^{67}Ga-DF-folate (Group 2). To competitively block tumor folate receptors three animals received 2.4 ± 1.0 mg folate intravenously ~5 minutes prior to ^{67}Ga-DF-folate administration (Group 3). Three different animals that received ^{67}Ga-DF-folate also received 3.5 ± 0.9 mg of folate intravenously approximately 1 hour before being sacrificed (Group 4). The tissue distribution of the tracers was periodically monitored by gamma scintigraphy to qualitatively assess tumor uptake of tracer and tumor-background contrast. Tumor uptake was evident at one hour post-injection, and by 3-4 hours post-injection the tracer initially present in the liver had substantially cleared into the intestines. At 4-4.5 hours following administration of the ^{67}Ga-radiopharmaceuticals the anesthetized animals were sacrificed by decapitation and the tumor and major organs removed, weighed, and stored until ^{67}Ga had decayed to levels suitable for counting. The biodistribution of tracer in each sample was calculated as both a percentage of the injected dose per organ and as a

percentage of the injected dose per gram of tissue (wet weight), using counts from a weighed and approximately diluted sample of the original injected for reference.

A summary of the biodistribution data for the ^{67}Ga-labeled deferoxamine-folate conjugate plus the ^{67}Ga-DF and ^{67}Ga-citrate reference tracers is presented in Tables 3 and 4. The data is also presented in bar graph form in Figs. 8 and 9. Fig. 8 illustrates the percent injected dose ^{67}Ga-radiotracer per gram tumor. Fig. 9 illustrates the ratio of ^{67}Ga-radiotracer concentration in tumor tissue compared to blood (% of injected dose per gram wet weight) at 4 to 4.5 hours post-injection. For both Fig. 8 and 9, each bar represents the data from one animal. Group 1 was administered ^{67}Ga-deferoxamine-folate; Group 2 was administered ^{67}Ga-deferoxamine-folate to mice maintained on a high folate diet; Group 3 was administered folic acid (approximately 2.4 mg) prior to administration of ^{67}Ga-deferoxamine-folate; Group 4 was administered ^{67}Ga-deferoxamine-folate with a chase dose of folate one hour prior to sacrifice; Group 5 was administered ^{67}Ga-deferoxamine; Group 6 was administered ^{67}Ga-citrate.

Table 3. Biodistribution of ^{67}Ga-Radiotracers in Athymic Mice with Subcutaneous KB Cell Tumors

Percentage of Injected Dose per Gram of Tissue 4 Hours Following Intravenous Administration

	^{67}Ga-DF-Folate				^{67}Ga-DF	^{67}GA-citrate
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Blood	0.14 ± 0.004	0.019 ± 0.011	0.45 ± 0.16	0.046 ± 0.009	0.026 ± 0.009	13.5 ± 3.2
Heart	0.029 ± 0.004	0.024 ± 0.014	0.17 ± 0.05	0.025 ± 0.005	0.021 ± 0.002	3.4 ± 0.2
Lungs	0.038 ± 0.005	0.063 ± 0.001	0.38 ± 0.14	0.052 ± 0.010	0.054 ± 0.010	9.1 ± 2.7
Liver	0.46 ± 0.08	0.40 ± 0.16	0.87 ± 0.18	0.43 ± 0.03	0.082 ± 0.010	4.7 ± 0.3
Kidney	2.02 ± 0.32	3.5 ± 1.8	24.3 ± 1.6	1.79 ± 0.82	1.26 ± 0.19	4.6 ± 0.4
Muscle	0.044 ± 0.006	0.029 ± 0.023	0.13 ± 0.06	0.028 ± 0.005	0.037 ± 0.001	2.04 ± 0.29
Tumor	5.2 ± 1.5	1.0 ± 0.29	0.26 ± 0.09	2.22 ± 0.36	0.094 ± 0.004	10.9 ± 0.2
Tumor mass (g)	0.21 ± 0.07	0.20 ± 0.02	0.11 ± 0.07	0.22 ± 0.02	0.13 ± 0.09	0.20 ± 0.03
Animal mass (g)	28.1 ± 1.1	25.6 ± 2.5	27.9 ± 2.7	28.3 ± 0.6	27.8 ± 2.3	27.6 ± 2.0

Table 4. Tumor to Background Tissue Contrast Obtained with ^{67}Ga-Radiotracers in the Athymic Mouse Model

Tumor-to-Non-target Ratio 4 Hours Following Intravenous Administration of Radiotracer

	^{67}Ga-DF-Folate				^{67}Ga-DF	^{67}Ga-citrate
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Tumor/blood	409 ± 195	60 ± 18	0.64 ± 0.31	48 ± 5	3.9 ± 1.2	0.84 ±0.19
Tumor/muscle	124 ± 47	44 ± 24	2.3 ± 1.2	82 ± 16	2.56 ± 0.15	5.4 ± 0.7
Tumor/liver	11.4 ± 3.2	2.5 ± 0.3	0.29 ± 0.07	5.1 ± 0.5	1.16 ± 0.10	2.3 ± 0.2
Tumor/kidney	2.6 ± 0.9	0.31 ± 0.08	0.011 ± 0.004	1.4 ± 0.5	0.08 ± 0.01	2.4 ± 0.3

Example 33 - ^{67}GA-DEFEROXAMINE-FOLATE CONJUGATE DOSE ESCALATION STUDY

A ^{67}Ga-labeled deferoxamine-folate conjugate (DF-folate) dose escalation study was carried out using athymic mice bearing subcutaneous folate-receptor-positive human KB cell tumors. Each animal typically received 1-10 μCi of ^{67}Ga-labeled radiotracer *via* intravenous injection into the femoral vein while the animal was temporarily anesthetized by inhalation of diethyl ether. Injection volumes were ~100 μL per animal. The administered dose of radiopharmaceutical solution was determined by weighing the injection syringe (to 0.0001 g) on a calibrated electronic analytical balance before and after solution injection. Tumor-bearing athymic mice were maintained on a folate-deficient diet for approximately 3 weeks prior to radiotracer administration to reduce serum folate levels to near the normal range for human serum. When rats were used in these studies, they were maintained on normal rodent chow. At the specified times following administration of the ^{67}Ga-radiopharmaceuticals, the anesthetized animals were sacrificed by decapitation, and the tumor and selected tissues were removed, weighed, and counted in an automatic gamma counter to assay ^{67}Ga-radioactivity. The quantity of radiotracer in each sample was calculated as both a percentage of the injected dose per organ (%ID/organ) and as a percentage of the injected dose per gram of tissue wet weight (%ID/g), using for reference, contemporaneously acquired counts from a measured aliquot (1/100) of a measured mass of the original injectate. Tumor/non-target tissue ratios were calculated from the corresponding %ID/g values.

Male Athymic Mice (Harlan, Nu Nu Strain) were fed a folate-free diet starting eight days before 2.8 x 10^6 KB cells per animal were implanted subcutaneously within the

interscapular region. Approximately three weeks after implantation of the tumor cells ^{67}Ga-labeled DF-folate was administered *via* the femoral vein at DF-folate doses of 133 (Group A), 27 (Group B), 2.8 (Group C), 0.29 (Group D), and 0.030 (Group E) mg/kg body mass. All animals were sacrificed 4 hours following tracer injection for quantitation of ^{67}Ga-biodistribution.

The accumulated data for the biodistribution of ^{67}Ga-Deferoxamine-Folate in each of the Athymic Mice is presented in Tables 5A, 5B and 5C. Tables 5A and 5B summarize the percentage of injected radioisotope retained per organ and per gram of organ tissue, respectively. Table 5C summarizes the tumor to background tissue contrast obtained with the ^{67}Ga-radiotracers in the Athymic mice. Values shown represent the mean ± standard deviation of data from four animals (n = 3 for Group E).

Tumor uptake of ^{67}Ga-DF-folate decreased at doses above 0.29 mg/kg, presumably due to competitive receptor binding by unlabeled excess DF-folate, dropping from 8.5 ± 0.4 %ID/g tumor at the 0.29 mg/kg dose to only 0.96 ± 0.17 %ID/g tumor at the 133 mg/kg dose (Table 5B). Tumor/blood, tumor/liver, and tumor/kidney ratios were highest at the intermediate 2.8 mg/kg dose with values of 290 ± 60, 24 ± 7, and 0.8 ± 0.2, respectively (Table 5C). At all doses >20% of the tracer was cleared into the intestines, making the subsequent rate of gastrointestinal-transit a major factor in determining the time-frame on which it would be feasible to image abdominal tumors.

Table 5A

	Percentage of Injected ^{67}Ga Dose per Organ (Tissue) 4 Hours Following Intravenous Administration				
	Group A	Group B	Group C	Group D	Group E
Blood	0.32 ± 0.10	0.091 ± 0.033	0.037 ± 0.003	0.086 ± 0.012	0.15 ± 0.01
Heart	0.020 ± 0.013	0.009[†] ± 0.009	0.014 ± 0.003	0.047 ± 0.007	0.094 ± 0.014
Lungs	0.04 ± 0.016	0.26 ± 0.47	0.014 ± 0.005	0.037 ± 0.005	0.065 ± 0.008
Liver	8.6 ± 1.1	0.94 ± 0.87	0.39 ± 0.06	1.2 ± 0.8	1.33 ± 0.09
Spleen	0.084 ± 0.018	0.017 ± 0.007	0.008 ± 0.003	0.030 ± 0.025	0.034 ± 0.003
Kidney (one)	23.8 ± 4.9	3.1 ± 1.8	2.0 ± 0.3	8.1 ± 1.3	14.3 ± 1.0
Intestines & Contents	20.7 ± 3.2	46.7 ± 4.4	27.7 ± 5.6	24.4 ± 5.2	18.7 ± 1.0
Tumor	0.199 ± 0.099	0.95 ± 0.30	2.21 ± 0.41	2.93 ± 0.44	2.76 ± 0.74
Tumor mass (g)	*0.22 ± 0.13*	*0.26 ± 0.05*	*0.33 ± 0.07*	*0.35 ± 0.07*	*0.32 ± 0.08*
Animal mass (g)	*29.2 ± 3.1*	*28.4 ± 2.2*	*28.1 ± 1.5*	*28.3 ± 3.6*	*28.2 ± 1.7*
Df-Folate Dose (mg/kg body mass)	*133 ± 24*	*27 ± 2*	*2.8 ± 0.3*	*0.29 ± 0.05*	*0.030 ± 0.001*

Table 5B

	Percentage of Injected ^{67}Ga Dose per Gram of Tissue 4 Hours Following Intravenous Administration				
	Group A	Group B	Group C	Group D	Group E
Blood	0.20 ± 0.07	0.058 ± 0.019	0.024 ± 0.01	0.055 ± 0.002	0.098 ± 0.012
Heart	0.14 ± 0.08	0.062† ± 0.051	0.10 ± 0.02	0.33 ± 0.03	0.67 ± 0.09
Lungs	0.25 ± 0.10	0.38 ± 0.41	0.073 ± 0.016	0.19 ± 0.02	0.39 ± 0.01
Liver	6.2 ± 3.3	0.67 ± 0.63	0.30 ± 0.11	0.86 ± 0.55	1.10 ± 0.09
Spleen	0.41 ± 0.16	0.084 ± 0.031	0.037 ± 0.013	0.14 ± 0.11	0.172 ± 0.005
Kidney	67.7 ± 19.8	11.0 ± 5.9	8.4 ± 0.4	35.8 ± 2.8	60.9 ± 7.3
Intestines & Contents	13.7 ± 2.2	30.3 ± 6.0	18.1 ± 4.0	15.0 ± 1.7	12.1 ± 1.2
Muscle	0.55 ± 0.47	0.35 ± 0.07	0.38 ± 0.14	0.69 ± 0.10	0.96 ± 0.04
Tumor	0.96 ± 0.17	3.7 ± 1.4	6.9 ± 1.5	8.5 ± 0.4	8.7 ± 1.4
Tumor mass (g)	*0.22 ± 0.13*	*0.26 ± 0.05*	*0.33 ± 0.07*	*0.35 ± 0.07*	*0.32 ± 0.08*
Animal mass (g)	*29.2 ± 3.1*	*28.4 ± 2.2*	*28.1 ± 1.5*	*28.3 ± 3.6*	*28.2 ± 1.7*
Df-Folate Dose (mg/kg body mass)	*133 ± 24*	*27 ± 2*	*2.8 ± 0.3*	*0.29 ± 0.05*	*0.030 ± 0.001*

Table 5C

Tumor-to-Non-target Radiotracer Ratio 4 Hours Following Intravenous Administration

	Group A	Group B	Group C	Group D	Group E
Tumor/blood	5.3 ± 2.4	72 ± 40	289 ± 61	154 ± 3	89 ± 3
Tumor/liver	0.16 ± 0.07	8.1 ± 5.4	23.8 ± 6.9	11.4 ± 4.1	8.0 ± 2.0
Tumor/kidney	0.014 ± 0.005	0.54 ± 0.55	0.82 ± 0.16	0.24 ± 0.02	0.14 ± 0.02

Example 34 - SYNTHESIS OF AN ^{111}In-DTPA-FOLATE CONJUGATE

The DTPA-folate conjugate (Figure 10) was synthesized in two steps. In the first step, folate-ethylenediamine was synthesized, as a mixture of α-linked and γ-linked isomers, by reacting an activated ester of folate with ethylenediamine. In the second step, DTPA-folate was synthesized by reacting folate-ethylene diamine with DTPA anhydride. The "active" γ-linked isomer was then purified by preparative HPLC.

Conjugation Chemistry. Folic acid (USP grade) was dissolved in warm DMSO (40°C) under stirring. (All reaction vessels are shielded from light by wrapping with aluminum foil). *N*-hydroxysuccinimyl folate (NHS-folate), an activated ester of folic acid was then be prepared by adding 3 molar equivalent of *N*-hydroxysuccinimide (NHS) and 1.5 molar equivalent of dicyclohexylcarbodiimide (DCC). The reaction mixture was stirred for 4 hours at 40°C. The insoluble by-product, dicyclohexylurea, is then removed by centrifugation. Five molar equivalent ethylenediamine was then added and the reaction mixture was stirred for 4 hours at 40°C. The product, folate-ethylenediamine, was precipitated with 5 volumes of cold acetone/diethylether (2:3) and washed three times with cold acetone. The pellet was then dissolved in a small volume of 0.1 N HCl. Unreacted folic acid is insoluble at low pH and was removed by centrifugation.

The pH of the solution was adjusted to 8.2 using 1 M sodium carbonate, and two and half molar equivalent of DTPA anhydride was added. The reaction mixture was incubated at room temperature for 10 min, and then for another 5 min following pH-adjustment to 10 to ensure complete hydrolysis of the DTPA anhydride. The DTPA-folate product was then precipitated with 10 volumes of cold acetone. Excess DTPA was removed by separation on a

preparative reverse-phase C-18 HPLC run with water containing 0.1% trifluoroacetic acid. The γ-linked isomer was then separated from the α-linked isomer on another preparative reverse-phase C-18 HPLC run with 10 mM ammonium bicarbonate buffer containing 4% acetonitrile.

The ^{111}In-labeled DTPA-Folate conjugate was obtained in high yield by ligand exchange from ^{111}In-citrate as follows. 0.30 mg DTPA-Folate was dissolved in water (0.15 mL) and adjusted to pH 7 by addition of aqueous sodium hydroxide. An aqueous solution of ^{111}In-chloride (5.4 mCi in 54 μL dilute HCl; Mallinckrodt Medical, St. Louis, MO) was mixed with 0.20 mL 3% (w/v) sodium citrate pH 7.4. The resulting ^{111}In-citrate solution was then added to the aqueous solution of DTPA-Folate and mixed at room temperature. The radiochemical purity of the ^{111}In-DTPA-Folate was determined by thin layer chromatography on C-18 eluted with methanol and generally found to exceed 98% after incubation at room temperature for 30 minutes (^{111}In-DTPA-Folate R_f = 0.8; ^{111}In-citrate R_f = 0.0).

<u>Example 35</u> - BIODISTRIBUTION OF ^{111}In-DTPA-FOLATE CONJUGATE

A biodistribution study with normal rats was conducted as described in Example 33. Each rat was injected via the femoral vein with 0.268 ± 0.021 mg/ml ^{111}In-DTPA-Folate. The results from this study demonstrate that the intravenously administered ^{111}In-DTPA-Folate is rapidly excreted into the urine with only 3.7 ± 1.4% of the dose retained in the intestines 4 hours after injection. Tables 6A and 6B summarize the percentage of injected radioisotope retained per organ and per gram of organ tissue, respectively. Values shown represent the mean ± standard deviation of data from three animals, each weighing approximately 188 ± 7g.

Table 6A

	Percentage of Injected ^{111}In Dose per Organ (Tissue)	
	4 hours	24 hours
Blood	0.078 ± 0.005	0.029 ± 0.006
Heart	0.021 ± 0.002	0.017 ± 0.002
Lungs	0.024 ± 0.003	0.022 ± 0.001
Liver	0.25 ± 0.01	0.17 ± 0.01
Spleen	0.019 ± 0.004	0.017 ± 0.003
Kidneys (two)	13.5 ± 1.4	12.1 ± 1.3
Intestines & Contents	3.7 ± 1.4	1.2 ± 0.3
Bladder & Contents	0.39 ± 0.63	0.09 ± 0.14

Table 6B

	Percentage of Injected ^{111}In Dose per Gram of Tissue	
	4 hours	24 hours
Blood	0.0059 ± 0.0006	0.0022 ± 0.0003
Heart	0.030 ± 0.005	0.025 ± 0.002
Lungs	0.023 ± 0.003	0.019 ± 0.002
Liver	0.033 ± 0.003	0.022 ± 0.001
Spleen	0.029 ± 0.001	0.028 ± 0.006
Kidney	8.9 ± 0.8	8.2 ± 0.9

Example 36 - BIODISTRIBUTION OF ^{111}In-DTPA-FOLATE CONJUGATE IN ATHYMIC MICE HAVING HUMAN KB CELL TUMORS

The biodistribution of ^{111}In-DTPA-Folate was determined in athymic mice bearing subcutaneously-implanted human KB cell tumors (Tables 7A and 7B). Three groups, each consisting of four animals, received the ^{111}In-DTPA-Folate conjugate intravenously as follows: The first group received only the radioisotope conjugate; the second group received the radioisotope conjugate and a simultaneous intravenous dose of folic acid (205 ± 18 µmol/kg Folate was co-injected with ^{111}In-DTPA-Folate to competitively block the folate receptor); and the third group received a chase dose of folic acid intravenously 3 hours following the ^{111}In-DTPA-Folate administration (203 ± 24 µmol/kg of folate was administered by i.v. injection 188 ± 10 minutes following administration of ^{111}In-DTPA-Folate to examine the competitive displacement of the ^{111}In-DTPA-Folate radiotracer). A fourth group, consisting of three mice received ^{111}In-DTPA intravenously (i.e. no folate targeting of the radioisotope) and, as an additional control experiment, a fifth group of four animals received ^{111}In-citrate intravenously.

Male athymic mice (Nu Nu strain) were injected subcutaneous within the intrascapular region with 1.8×10^6 KB tumor cells per animal as described in Example 33. The mice were initiated on a folate-free diet six days prior to implantation of the KB cells and two weeks after implantation of the tumor cells the radioisotope was injected. The ^{111}In-DTPA-Folate tracer was found to significantly concentrate in the KB tumors with tumor uptake of 1.0 ± 0.5% of the injected dose (3.1 ± 0.6 %ID per gram) at 4 hours post-injection. Tables 7A and 7B summarize the percentage of injected radioisotope retained per organ and per gram of organ tissue, respectively. Values shown represent the mean ± standard deviation of

data from four animals (*n* = 3 for ^{111}In-DTPA). Blood was assumed to account for 5.5% of total body mass. Tumor/Background Tissue ratios were based on corresponding %Injected Dose per Gram data.

Exceptional tumor/intestine contrast was obtained with ^{111}In-DTPA-Folate, due to efficient urinary clearance of the ^{111}In-tracer and a corresponding decrease in the fraction of ^{111}In cleared into the intestines via the hepatobiliary system. The specific involvement of the folate receptor in mediating the tumor uptake of ^{111}In-DTPA-Folate was demonstrated by the reduced tumor accumulation of ^{111}In-DTPA-Folate in the mice that received a simultaneous blocking dose of folic acid. Only a slight reduction of tumor accumulation of ^{111}In-DTPA-Folate was observed in the four mice that received a "chase" dose of folate intravenously 1 hour prior to sacrifice. This result indicates that the tumor-localized ^{111}In-DTPA-Folate tracer had largely been internalized by the tumor cells at the time of administration of the chase dose.

The control experiments demonstrate that, as expected, unconjugated ^{111}In-DTPA showed no tumor affinity (See Tables 7A and 7B). This contrasts with the administration of ^{111}In-citrate, which showed some tumor affinity but poor tumor/background tissue contrast (Tables 7A and 7B).

Table 7A

Percentage of Injected ^{111}In Dose per Organ (Tissue) 4 Hours Following Intravenous Administration	^{111}In-DTPA-Folate	^{111}In-DTPA-Folate + Folate Co-Injection	^{111}In-DTPA-Folate + Folate Chase @ 3 hours	^{111}In-DTPA	^{111}In-citrate
DTPA-Folate Dose (µmol/kg):	*2.77 ± 0.35*	*2.78 ± 0.24*	*2.87 ± 0.25*	-	-
Animal mass (g):	*29.0 ± 3.6*	*29.5 ± 2.2*	*29.2 ± 1.1*	*28.5 ± 3.5*	*30.3 ± 2.5*
Tumor mass (g):	*0.33 ± 0.15*	*0.31 ± 0.11*	*0.31 ± 0.18*	*0.21 ± 0.08*	*0.44 ± 0.25*
Blood:	0.014 ± 0.10	0.32 ± 0.17	0.041 ± 0.012	0.0056 ± 0.0005	16.5 ± 4.2
Heart:	0.0042 ± 0.013	0.013 ± 0.006	0.0048 ± 0.0012	0.0018 ± 0.0001	0.54 ± 0.16
Lungs:	0.018 ± 0.016	0.063 ± 0.017	0.021 ± 0.0007	0.011 ± 0.0002	3.1 ± 1.1
Liver:	0.15 ± 1.1	3.88 ± 0.19	0.12 ± 0.02	0.109 ± 0.007	7.2 ± 2.3
Spleen:	0.0061 ± 0.018	0.11 ± 0.02	0.0066 ± 0.0008	0.0049 ± 0.0008	1.2 ± 0.6
Kidneys (two):	1.61 ± 0.25	19.9 ± 8.1	1.33 ± 0.18	0.43 ± 0.04	7.0 ± 1.4
Intestines & Contents:	0.96 ± 0.19	1.9 ± 0.7	0.72 ± 0.16	0.43 ± 0.25	6.5 ± 3.2
Tumor:	1.01 ± 0.50	0.11 ± 0.08	0.64 ± 0.19	0.0091 ± 0.0033	1.7 ± 1.2
Tumor/Blood:	*346 ± 101*	*1.7 ± 0.5*	*96.8 ± 40.6*	*12.5 ± 0.5*	*0.38 ± 0.08*
Tumor/Liver:	*31.1 ± 9.7*	*0.12 ± 0.06*	*28.8 ± 10.9*	*0.54 ± 0.02*	*0.72 ± 0.04*
Tumor/Kidney:	*1.00 ± 0.34*	*0.010 ± 0.002*	*1.22 ± 0.46*	*0.05 ± 0.01*	*0.28 ± 0.07*
Tumor/Muscle:	*33.3 ± 9.8*	*1.1 ± 0.2*	*41.4 ± 17.6*	*2.4 ± 0.5*	*2.4 ± 0.4*

Table 7B

	Percentage of Injected ^{111}In Dose per Gram of Tissue 4 Hours Following Intravenous Administration				
	^{111}In-DTPA-Folate	^{111}In-DTPA-Folate + Folate Co-Injection	^{111}In-DTPA-Folate + Folate Chase @ 3 hours	^{111}In-DTPA	^{111}In-citrate
DTPA-Folate Dose (μmol/kg):	*2.77 ± 0.35*	*2.78 ± 0.24*	*2.87 ± 0.25*	-	-
Animal mass (g):	*29.0 ± 3.6*	*29.5 ± 2.2*	*29.2 ± 1.1*	*28.5 ± 3.5*	*30.3 ± 2.5*
Tumor mass (g):	*0.33 ± 0.15*	*0.31 ± 0.11*	*0.31 ± 0.18*	*0.21 ± 0.08*	*0.44 ± 0.25*
Blood:	0.0093 ± 0.0029	0.20 ± 0.10	0.026 ± 0.008	0.0036 ± 0.0004	9.9 ± 2.3
Heart:	0.032 ± 0.008	0.10 ± 0.04	0.033 ± 0.005	0.013 ± 0.001	3.7 ± 1.0
Lungs:	0.048 ± 0.005	0.21 ± 0.09	0.050 ± 0.010	0.026 ± 0.001	6.5 ± 2.0
Liver:	0.10 ± 0.02	2.8 ± 0.3	0.084 ± 0.013	0.084 ± 0.011	5.1 ± 1.3
Spleen:	0.038 ± 0.011	0.58 ± 0.14	0.039 ± 0.002	0.024 ± 0.005	5.3 ± 1.8
Kidney:	3.2 ± 0.6	31.2 ± 11.9	1.97 ± 0.15	0.85 ± 0.07	13.0 ± 1.3
Intestines & Contents:	0.45 ± 0.16	1.1 ± 0.5	0.38 ± 0.08	0.22 ± 0.13	3.3 ± 1.4
Muscle:	0.094 ± 0.012	0.28 ± 0.08	0.059 ± 0.006	0.02 ± 0.01	1.6 ± 0.4
Tumor:	3.1 ± 0.6	0.31 ± 0.13	2.38 ± 0.85	0.045 ± 0.005	3.6 ± 0.8
Tumor/Blood:	*346 ± 101*	*1.73 ± 0.45*	*96.8 ± 40.6*	*12.5 ± 0.5*	*0.38 ± 0.08*
Tumor/Liver:	*31.1 ± 9.7*	*0.12 ± 0.06*	*28.8 ± 10.9*	*0.54 ± 0.02*	*0.72 ± 0.04*
Tumor/Kidney:	*1.00 ± 0.34*	*0.010 ± 0.002*	*1.22 ± 0.46*	*0.05 ± 0.01*	*0.28 ± 0.07*
Tumor/Muscle:	*33.3 ± 9.8*	*1.10 ± 0.23*	*41.4 ± 17.6*	*2.4 ± 0.5*	*2.4 ± 0.4*

Example 37 - DOSE ESCALATION/COMPETITIVE BINDING STUDY IN ATHYMIC MICE HAVING HUMAN KB CELL TUMORS

A dose escalation study was conducted using athymic mice bearing subcutaneous folate-receptor-positive human KB cell tumors. The ^{111}In-DTPA-Folate radiopharmaceutical was administered *via* the femoral vein at DTPA-folate doses of approximately 45 nmol/kg body mass, and free folic acid was co-injected at doses of approximately 0.0 (Group A), 0.3 (Group B), 3 (Group C), 30 (Group D), or 300 (Group E) μmol/kg (Tables 8A and 8B). All animals were sacrificed 4 hours following radiotracer injection. Values shown represent the mean ± standard deviation of data from four animals. Blood was assumed to account for 5.5% of total body mass. Tumor/Background Tissue ratios were based on corresponding %Injected Dose per Gram data.

Male athymic mice (Nu Nu strain) were initiated on a folate-free diet seven days before implantation of 1.8 x 10^6 KB cells per animal. Each mouse was injected with an equivalent amount of ^{111}In-DTPA-Folate approximately two weeks after injection of the KB cells, and the animals were sacrificed four hours after administration of the ^{111}In-DTPA-Folate.

Tumor uptake of ^{111}In-DTPA-Folate (calculated as a percentage of the injected dose or as a percentage of the injected dose per gram of tissue) decreased at folic acid doses above 0.3 μmol/kg, presumably due to competitive receptor binding by the unlabeled free folate. Tumor uptake of ^{111}In-DTPA-Folate was 6.9 ± 1.7 and 5.1 ± 0.3 %ID/g at folic acid doses of 0.0 and 0.3 μmol/kg, respectively, but dropped to only 1.7 ± 0.2, 0.8 ± 0.3, and 1.1 ± 0.2 %ID/g tumor at the 3, 30, and 300 μmol/kg doses of folic acid, respectively (Table 8B). Tumor/blood, tumor/liver, and tumor/muscle contrast was highest at the intermediate 0.3 μmol/kg folic acid dose, where tumor/non-target tissue

ratios of 210 ± 32 (tumor/blood), 33 ± 4 (tumor/liver), and 36 ± 5 (tumor/muscle) were observed (Table 8B).

Tumor/kidney contrast was highest at the 3 μmol/kg folic acid dose, where a tumor/kidney ratio of 0.68 ± 0.13 was observed (Table 8B). Kidney uptake of the ^{111}In-DTPA-Folate radiotracer dropped from 82 ± 9 %ID/g, when no folic acid was included in the injectate, to 16 ± 1 and 2.6 ± 0.2 %ID/g at folic acid doses of 0.3 and 3 μmol/kg, respectively; this indicates that the administered folic acid and ^{111}In-DTPA-Folate were competitively binding to low levels of folate receptor known to be present in the proximal tubule of the kidney (Table 8B). The observed increase in kidney levels of indium-111 when the folic acid dose is increased from 3 μmol/kg to 30 and 300 μmol/kg is believed to result from precipitation of folic acid when the latter was concentrated and acidified in the urine (resulting in mechanical obstruction of urine flow from the kidney to the bladder). At all doses <2% of the radiotracer was cleared into the intestines, minimizing the potential impact of GI-radioactivity with regard to the feasibility of imaging abdominal tumors.

Table 8A

	Percentage of Injected ^{111}In Dose per Organ (Tissue) 4 Hours Following Intravenous Administration				
	A ^{111}In-DTPA-Folate	B ^{111}In-DTPA-Folate + Folic Acid	C ^{111}In-DTPA-Folate + Folic Acid	D ^{111}In-DTPA-Folate + Folic Acid	E ^{111}In-DTPA-Folate + Folic Acid
Folic Acid Dose (μmol/kg):	*0 ± 0*	*0.305 ± 0.024*	*3.29 ± 0.28*	*32.2 ± 1.6*	*321 ± 47*
DTPA-Folate Dose (nmol/kg):	*46.9 ± 3.7*	*41.2 ± 3.3*	*44.4 ± 3.8*	*43.4 ± 2.2*	*43.5 ± 6.4*
Animal mass (g):	*27.2 ± 1.7*	*27.6 ± 4.6*	*29.1 ± 2.3*	*29.9 ± 1.4*	*28.9 ± 2.1*
Tumor mass (g):	*0.38 ± 0.19*	*0.60 ± 0.21*	*0.55 ± 0.14*	*0.69 ± 0.06*	*0.57 ± 0.22*
Blood:	0.092 ± 0.012	0.038 ± 0.010	0.034 ± 0.003	0.65 ± 0.47	1.7 ± 0.4
Heart:	0.18 ± 0.03	0.010 ± 0.002	0.0044 ± 0.0023	0.025 ± 0.014	0.060 ± 0.016
Lungs:	0.34 ± 0.02	0.037 ± 0.003	0.026 ± 0.002	0.13 ± 0.05	0.33 ± 0.02
Liver:	2.07 ± 0.25	0.21 ± 0.02	0.18 ± 0.13	0.66 ± 0.28	0.97 ± 0.20
Spleen:	0.053 ± 0.017	0.013 ± 0.002	0.012 ± 0.008	0.053 ± 0.023	0.11 ± 0.02
Kidney (one):	17.8 ± 2.2	3.6 ± 0.4	0.57 ± 0.06	12.9 ± 4.2	39.1 ± 3.0
Intestines & Contents:	1.9 ± 0.1	0.89 ± 0.06	1.1 ± 0.1	3.6 ± 1.0	2.6 ± 0.3
Testes	0.25 ± 0.05	0.043 ± 0.005	0.013 ± 0.004	0.034 ± 0.012	0.058 ± 0.008
Tumor:	2.39 ± 0.80	3.13 ± 1.23	0.93 ± 0.17	0.52 ± 0.20	0.61 ± 0.22
Tumor/Blood:	*114. ± 32*	*210 ± 32*	*82 ± 15*	*2.4 ± 0.9*	*1.0 ± 0.1*
Tumor/Liver:	*3.9 ± 1.3*	*32.9 ± 4.3*	*15.1 ± 6.1*	*1.58 ± 0.15*	*1.4 ± 0.2*
Tumor/Kidney:	*0.085 ± 0.021*	*0.33 ± 0.03*	*0.68 ± 0.13*	*0.017 ± 0.003*	*0.011 ± 0.003*
Tumor/Muscle:	*3.1 ± 0.7*	*35.7 ± 4.9*	*30.8 ± 16.4*	*2.68 ± 0.75*	*1.1 ± 0.2*

Table 8B

	Percentage of Injected ^{111}In Dose per Gram of Tissue 4 Hours Following Intravenous Administration				
	A	B	C	D	E
	^{111}In-DTPA-Folate	^{111}In-DTPA-Folate + Folic Acid	^{111}In-DTPA-Folate + Folic Acid	^{111}In-DTPA-Folate + Folic Acid	^{111}In-DTPA-Folate + Folic Acid
Folic Acid Dose (μmol/kg):	*0 ± 0*	*0.305 ± 0.024*	*3.29 ± 0.28*	*32.2 ± 1.6*	*321 ± 47*
DTPA-Folate Dose (nmol/kg):	*46.9 ± 3.7*	*41.2 ± 3.3*	*44.4 ± 3.8*	*43.4 ± 2.2*	*43.5 ± 6.4*
Animal mass (g):	*27.2 ± 1.7*	*27.6 ± 4.6*	*29.1 ± 2.3*	*29.9 ± 1.4*	*28.9 ± 2.1*
Tumor mass (g):	*0.38 ± 0.19*	*0.60 ± 0.21*	*0.55 ± 0.14*	*0.69 ± 0.06*	*0.57 ± 0.22*
Blood:	0.062 ± 0.009	0.025 ± 0.003	0.021 ± 0.002	0.40 ± 0.29	1.05 ± 0.26
Heart:	1.4 ± 0.2	0.069 ± 0.009	0.033 ± 0.016	0.18 ± 0.12	0.44 ± 0.12
Lungs:	0.94 ± 0.17	0.077 ± 0.018	0.057 ± 0.016	0.38 ± 0.22	0.89 ± 0.24
Liver:	1.82 ± 0.26	0.16 ± 0.02	0.14 ± 0.08	0.49 ± 0.21	0.80 ± 0.21
Spleen:	0.28 ± 0.06	0.060 ± 0.009	0.058 ± 0.034	0.25 ± 0.11	0.52 ± 0.12
Kidney:	82.1 ± 8.5	15.5 ± 0.8	2.6 ± 0.2	43.5 ± 14.1	102.0 ± 4.7
Intestines & Contents:	0.98 ± 0.20	0.43 ± 0.06	0.54 ± 0.08	1.7 ± 0.4	1.4 ± 0.3
Muscle:	2.3 ± 0.3	0.15 ± 0.02	0.068 ± 0.035	0.29 ± 0.12	0.94 ± 0.07
Testes	1.49 ± 0.38	0.25 ± 0.03	0.072 ± 0.015	0.21 ± 0.08	0.35 ± 0.09
Tumor:	6.93 ± 1.66	5.16 ± 0.28	1.74 ± 0.23	0.77 ± 0.34	1.08 ± 0.22
Tumor/Blood:	*114 ± 32*	*210 ± 32*	*82 ± 15*	*2.4 ± 0.9*	*1.0 ± 0.1*
Tumor/Liver:	*3.9 ± 1.3*	*32.9 ± 4.3*	*15.1 ± 6.1*	*1.58 ± 0.15*	*1.37 ± 0.15*
Tumor/Kidney:	*0.085 ± 0.021*	*0.33 ± 0.03*	*0.68 ± 0.13*	*0.017 ± 0.003*	*0.011 ± 0.003*
Tumor/Muscle:	*3.1 ± 0.7*	*35.7 ± 4.9*	*30.8 ± 16.4*	*2.68 ± 0.75*	*1.14 ± 0.16*

Example 38 - BIODISTRIBUTION OF ^{111}In-DTPA-FOLATE CONJUGATE IN ATHYMIC MICE HAVING HUMAN KB CELL TUMORS

A biodistribution study of ^{111}In-DTPA-Folate was conducted utilizing athymic mice subcutaneous injected with human KB cell tumors. The ^{111}In-DTPA-Folate radiopharmaceutical was administered by femoral vein injection using a formulation containing DTPA-folate at a dose of approximately 30 µg/kg and folic acid dihydrate at a dose of approximately 170 µg/kg. The mice, in groups of four, were sacrificed at 1 minute, 5 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 24 hours, or 48 hours post-injection for quantitation of radiotracer biodistribution (Tables 9A and 9B). Tumor uptake of ^{111}In peaked by 30 minutes post-injection and remained fairly constant thereafter. The tumor radiotracer levels (^{111}In dose/g) over time were as follows:

Tumor %ID/g	Time (post injection)
3.9 ± 2.0	1 minute
3.9 ± 0.8	5 minutes
6.3 ± 1.7	30 minutes
5.8 ± 1.3	1 hour
6.9 ± 0.9	2 hours
7.3 ± 1.8	4 hours
5.8 ± 1.9	24 hours
3.9 ± 0.5	48 hours

By 30 minutes post-injection, tumor/non-target tissue ratios appear sufficient to allow tumor imaging with good tumor/background contrast (Tables 9A and 9B).

TABLE 9A (PART 1 OF 3)

	1 minute	5 minutes
Dose Folic Acid Dihydrate (μg/kg):	*167 ± 26*	*176 ± 19*
Dose DTPA-Folate (μg/kg):	*29.7 ± 4.5*	*31.3 ± 3.3*
Animal mass (g):	*26.2 ± 3.3*	*25.4 ± 2.1*
Tumor mass (g):	*0.19 ± 0.13*	*0.29 ± 0.10*
Blood:	18.2 ± 1.9	8.8 ± 0.9
Thyroid:	0.16 ± 0.04	0.082 ± 0.017
Heart:	0.50 ± 0.06	0.26 ± 0.02
Lungs:	3.60 ± 0.77	1.77 ± 0.21
Liver:	3.30 ± 0.44	2.03 ± 0.18
Spleen:	0.43 ± 0.09	0.24 ± 0.03
Kidney (one):	7.0 ± 1.2	3.3 ± 0.9
Adrenal (one):	0.025 ± 0.003	0.013 ± 0.002
Stomach:	0.81 ± 0.12	0.58 ± 0.04
Intestines:	5.3 ± 0.3	2.8 ± 0.2
Pancreas:	0.28 ± 0.07	0.18 ± 0.02
Ovary (one):	0.038 ± 0.017	0.030 ± 0.006
Uterus:	0.54 ± 0.40	0.19 ± 0.05
Red Muscle:	18.7 ± 3.1	13.8 ± 2.7
White Muscle:	22.6 ± 4.8	23.7 ± 6.7
Brain:	0.15 ± 0.01	0.081 ± 0.008
Bladder & Contents:	0.12 ± 0.06	0.38 ± 0.18
Tumor:	0.67 ± 0.31	1.11 ± 0.37
Tumor / Blood :	*0.31 ± 0.15*	*0.63 ± 0.15*
Tumor / Liver :	*1.19 ± 0.56*	*2.15 ± 0.55*
Tumor / Kidney :	*0.10 ± 0.06*	*0.23 ± 0.07*
Tumor / Muscle :	*2.4 ± 1.3*	*3.13 ± 0.96*

TABLE 9A (PART 2 OF 3)

30 minutes	1 hour	2 hours
172 ± 18	*189 ± 20*	*164 ± 25*
30.4 ± 3.1	*33.6 ± 3.5*	*29.2 ± 4.5*
25.2 ± 1.9	*24.7 ± 3.0*	*26.9 ± 3.1*
0.13 ± 0.04	*0.23 ± 0.05*	*0.25 ± 0.09*
0.78 ± 0.26	0.13 ± 0.04	0.046 ± 0.005
0.009 ± 0.003	0.0019 ± 0.0012	0.0017 ± 0.0006
0.032 ± 0.010	0.0082 ± 0.0017	0.0059 ± 0.0014
0.24 ± 0.04	0.073 ± 0.013	0.015 ± 0.002
0.59 ± 0.06	0.33 ± 0.07	0.53 ± 0.63
0.028 ± 0.005	0.011 ± 0.006	0.0080 ± 0.0010
1.7 ± 0.2	1.4 ± 0.3	1.55 ± 0.09
0.0034 ± 0.0003	0.0020 ± 0.0010	0.0019 ± 0.0005
0.14 ± 0.07	0.045 ± 0.010	0.032 ± 0.014
0.78 ± 0.13	0.63 ± 0.13	0.95 ± 0.47
0.032 ± 0.011	0.0084 ± 0.0029	0.0084 ± 0.0033
0.0083 ± 0.0024	0.0030 ± 0.0009	0.0024 ± 0.0004
0.063 ± 0.056	0.011 ± 0.008	0.0076 ± 0.0013
1.75 ± 0.40	0.89 ± 0.91	0.44 ± 0.17
3.39 ± 0.74	0.82 ± 0.28	0.65 ± 0.08
0.024 ± 0.003	0.016 ± 0.002	0.020 ± 0.002
0.33 ± 0.005	0.010 ± 0.007	0.17 ± 0.33
0.85 ± 0.36	1.32 ± 0.31	1.66 ± 0.41
12.1 ± 4.6	*69.8 ± 39.7*	*227 ± 64*
12.0 ± 3.2	*19.7 ± 7.3*	*29.5 ± 18.9*
0.67 ± 0.16	*0.79 ± 0.29*	*0.74 ± 0.20*
38.8 ± 8.5	*118 ± 67*	*197 ± 67*

TABLE 9A (PART 3 OF 3)

4 hours	24 hours	48 hours
152 ± 21	*173 ± 20*	*175 ± 13*
26.9 ± 3.7	*30.7 ± 3.6*	*31.1 ± 2.3*
26.2 ± 1.5	*24.8 ± 2.0*	*24.7 ± 1.2*
0.25 ± 0.24	*0.27 ± 0.07*	*0.29 ± 0.27*
0.035 ± 0.007	0.020 ± 0.006	0.0092 ± 0.0021
0.00093 ± 0.00021	0.00081 ± 0.00041	0.0015 ± 0.0007
0.0065 ± 0.0017	0.0045 ± 0.0006	0.0045 ± 0.0011
0.028 ± 0.002	0.016 ± 0.001	0.012 ± 0.001
0.14 ± 0.05	0.086 ± 0.013	0.074 ± 0.021
0.0085 ± 0.0020	0.0063 ± 0.0011	0.0070 ± 0.0034
1.9 ± 0.1	1.4 ± 0.35	1.10 ± 0.23
0.0028 ± 0.0003	0.0014 ± 0.0009	0.0014 ± 0.0004
0.020 ± 0.006	0.017 ± 0.013	0.015 ± 0.005
0.78 ± 0.18	0.28 ± 0.11	0.12 ± 0.02
0.0077 ± 0.0022	0.0057 ± 0.0021	0.0059 ± 0.0019
0.0027 ± 0.0008	0.0019 ± 0.0010	0.0014 ± 0.0003
0.010 ± 0.002	0.010 ± 0.0051	0.0087 ± 0.0055
0.31 ± 0.05	0.098 ± 0.017	0.22 ± 0.10
0.70 ± 0.22	0.41 ± 0.09	0.52 ± 0.12
0.019 ± 0.003	0.014 ± 0.001	0.012 ± 0.004
0.0044 ± 0.0023	0.0054 ± 0.0013	0.0027 ± 0.0010
1.61 ± 1.44	1.49 ± 0.39	1.04 ± 0.79
301 ± 65	*384 ± 48*	*604 ± 183*
69 ± 37	*75 ± 23*	*62.4 ± 23.6*
0.71 ± 0.20	*0.75 ± 0.25*	*0.65 ± 0.15*
269 ± 90	*646 ± 324*	*247 ± 194*

TABLE 9B (PART 1 OF 3)

	1 minute	5 minutes
Dose Folic Acid Dihydrate (μg/kg):	*167 ± 26*	*176 ± 19*
Dose DTPA-Folate (μg/kg):	*29.7 ± 4.5*	*31.3 ± 3.3*
Animal mass (g):	*26.2 ± 3.3*	*25.4 ± 2.1*
Tumor mass (g):	*0.19 ± 0.13*	*0.29 ± 0.10*
Blood:	12.7 ± 0.7	6.3 ± 0.7
Heart:	4.2 ± 0.2	2.2 ± 0.2
Lungs:	8.8 ± 1.3	4.4 ± 0.5
Liver:	3.3 ± 0.2	1.9 ± 0.3
Spleen:	2.9 ± 0.4	1.5 ± 0.3
Kidney (one):	39.7 ± 9.6	17.9 ± 4.4
Adrenal (one):	4.3 ± 1.0	1.7 ± 0.4
Stomach:	2.3 ± 0.9	1.14 ± 0.29
Intestines:	2.9 ± 0.4	1.6 ± 0.3
Pancreas:	2.1 ± 0.1	1.28 ± 0.21
Ovary (one):	2.1 ± 0.5	2.16 ± 0.89
Uterus:	6.0 ± 2.9	4.6 ± 0.95
Red Muscle:	1.7 ± 0.2	1.3 ± 0.3
White Muscle:	2.1 ± 0.3	2.2 ± 0.5
Skin:	3.9 ± 0.7	4.7 ± 0.6
Bone (Femur):	2.3 ± 0.4	
Bone (Tibia):	2.1 ± 0.3	
Brain:	0.4 ± 0.1	0.22 ± 0.02
Bladder & Contents:	7.2 ± 3.6	22.8 ± 12.7
Tumor:	3.9 ± 2.0	3.9 ± 0.8
Tumor/Blood :	*0.31 ± 0.15*	*0.63 ± 0.15*
Tumor/Liver :	*1.19 ± 0.56*	*2.15 ± 0.55*
Tumor/Kidney :	*0.10 ± 0.06*	*0.23 ± 0.07*
Tumor/Muscle :	*2.4 ± 1.3*	*3.13 ± 0.96*

TABLE 9B (Part 2 of 3)

30 minutes	1 hour	2 hours
172 ± 18	*189 ± 20*	*164 ± 25*
30.4 ± 3.1	*33.6 ± 3.5*	*29.2 ± 4.5*
25.2 ± 1.9	*24.7 ± 3.0*	*26.9 ± 3.1*
0.13 ± 0.04	*0.23 ± 0.05*	*0.25 ± 0.09*
0.56 ± 0.18	0.094 ± 0.028	0.031 ± 0.006
0.25 ± 0.07	0.068 ± 0.012	0.044 ± 0.012
0.70 ± 0.09	0.19 ± 0.05	0.077 ± 0.047
0.53 ± 0.07	0.31 ± 0.04	0.45 ± 0.52
0.19 ± 0.04	0.060 ± 0.010	0.054 ± 0.007
9.4 ± 0.5	7.6 ± 1.4	9.6 ± 1.9
0.53 ± 0.25	0.32 ± 0.12	0.25 ± 0.18
0.30 ± 0.06	0.084 ± 0.035	0.040 ± 0.021
0.42 ± 0.06	0.35 ± 0.04	0.50 ± 0.31
0.22 ± 0.05	0.069 ± 0.016	0.058 ± 0.016
0.48 ± 0.15	0.16 ± 0.03	0.18 ± 0.06
0.57 ± 0.14	0.16 ± 0.03	0.14 ± 0.04
0.16 ± 0.03	0.089 ± 0.094	0.039 ± 0.017
0.32 ± 0.07	0.078 ± 0.023	0.059 ± 0.013
0.77 ± 0.32	0.24 ± 0.07	0.18 ± 0.05
	0.063 ± 0.014	
	0.071 ± 0.034	
0.06 ± 0.01	0.040 ± 0.004	0.051 ± 0.004
2.17 ± 0.80	0.69 ± 0.18	9.3 ± 17.8
6.34 ± 1.74	5.82 ± 1.32	6.87 ± 0.93
12.1 ± 4.6	*69.8 ± 39.7*	*227 ± 64*
12.0 ± 3.2	*19.7 ± 7.3*	*29.5 ± 18.9*
0.67 ± 0.16	*0.79 ± 0.29*	*0.74 ± 0.20*
38.8 ± 8.5	*118 ± 67*	*197 ± 67*

TABLE 9B (PART 3 OF 3)

4 hours	24 hours	48 hours
152 ± 21	*173 ± 20*	*175 ± 13*
26.9 ± 3.7	*30.7 ± 3.6*	*31.1 ± 2.3*
26.2 ± 1.5	*24.8 ± 2.0*	*24.7 ± 1.2*
0.25 ± 0.24	*0.27 ± 0.07*	*0.29 ± 0.27*
0.024 ± 0.006	0.015 ± 0.004	0.0068 ± 0.0018
0.051 ± 0.013	0.037 ± 0.003	0.036 ± 0.009
0.069 ± 0.008	0.041 ± 0.003	0.032 ± 0.002
0.12 ± 0.05	0.077 ± 0.003	0.068 ± 0.020
0.046 ± 0.004	0.042 ± 0.004	0.041 ± 0.021
10.3 ± 0.7	7.8 ± 1.1	6.1 ± 0.9
0.47 ± 0.11	0.23 ± 0.14	0.17 ± 0.05
0.052 ± 0.023	0.053 ± 0.020	0.035 ± 0.015
0.42 ± 0.10	0.16 ± 0.05	0.063 ± 0.011
0.054 ± 0.018	0.046 ± 0.008	0.041 ± 0.009
0.22 ± 0.07	0.12 ± 00.68	0.16 ± 0.02
0.15 ± 0.03	0.14 ± 0.072	0.14 ± 0.08
0.028 ± 0.005	0.0095 ± 0.0020	0.021 ± 0.010
0.064 ± 0.021	0.039 ± 0.006	0.051 ± 0.012
0.20 ± 0.06	0.14 ± 0.01	0.12 ± 0.02
	0.054 ± 0.021	0.037 ± 0.015
	0.051 ± 0.007	0.038 ± 0.006
0.047 ± 0.007	0.035 ± 0.005	0.032 ± 0.010
0.26 ± 0.12	0.32 ± 0.08	0.15 ± 0.02
7.25 ± 1.82	5.79 ± 1.90	3.87 ± 0.53
301 ± 65	*384 ± 48*	*604 ± 183*
69 ± 37	*75 ± 23*	*62.4 ± 23.6*
0.71 ± 0.20	*0.75 ± 0.25*	*0.65 ± 0.15*
269 ± 90	*646 ± 324*	*247 ± 194*

CLAIMS:

1. A method for detecting tumors in a vertebrate species, said method comprising the steps of administering to said vertebrate species a composition comprising an diagnostic agent complexed with a ligand selected from the group consisting of folate or folate receptor-binding analogs of folate, in a pharmaceutically acceptable carrier, excipient or diluent, and monitoring the biodistribution of said complex.

2. The complex of claim 1 wherein the complex is formed by covalent, ionic or hydrogen bonding of the ligand to the diagnostic agent either directly or indirectly through a linking group.

3. The method of claim 1 wherein the diagnostic agent is complexed to the gamma-carboxylate of said folate.

4. The method of claim 1 wherein the imaging agent is a radionuclide.

5. The method of claim 1 wherein the radionuclide is selected from the group consisting of isotopes of gallium, indium, copper, technetium, or rhenium.

6. The method of claim 1 wherein said composition is administered by intravenous injection.

7. In a method for imaging tumors in a vertebrate species by administering a compound capable of being detected *in vivo*, the improvement which comprises complexing said compound with a ligand selected from the group consisting of folate or folate receptor-binding analogs of folate.

8. A complex for imaging tumor cells *in vivo*, said complex comprising a folate molecule or a folate receptor-binding analog of folate; a deferoxamine molecule covalently linked to said folate molecule or said folate

receptor-binding analog of folate; and a radionuclide chelated to said deferoxamine molecule.

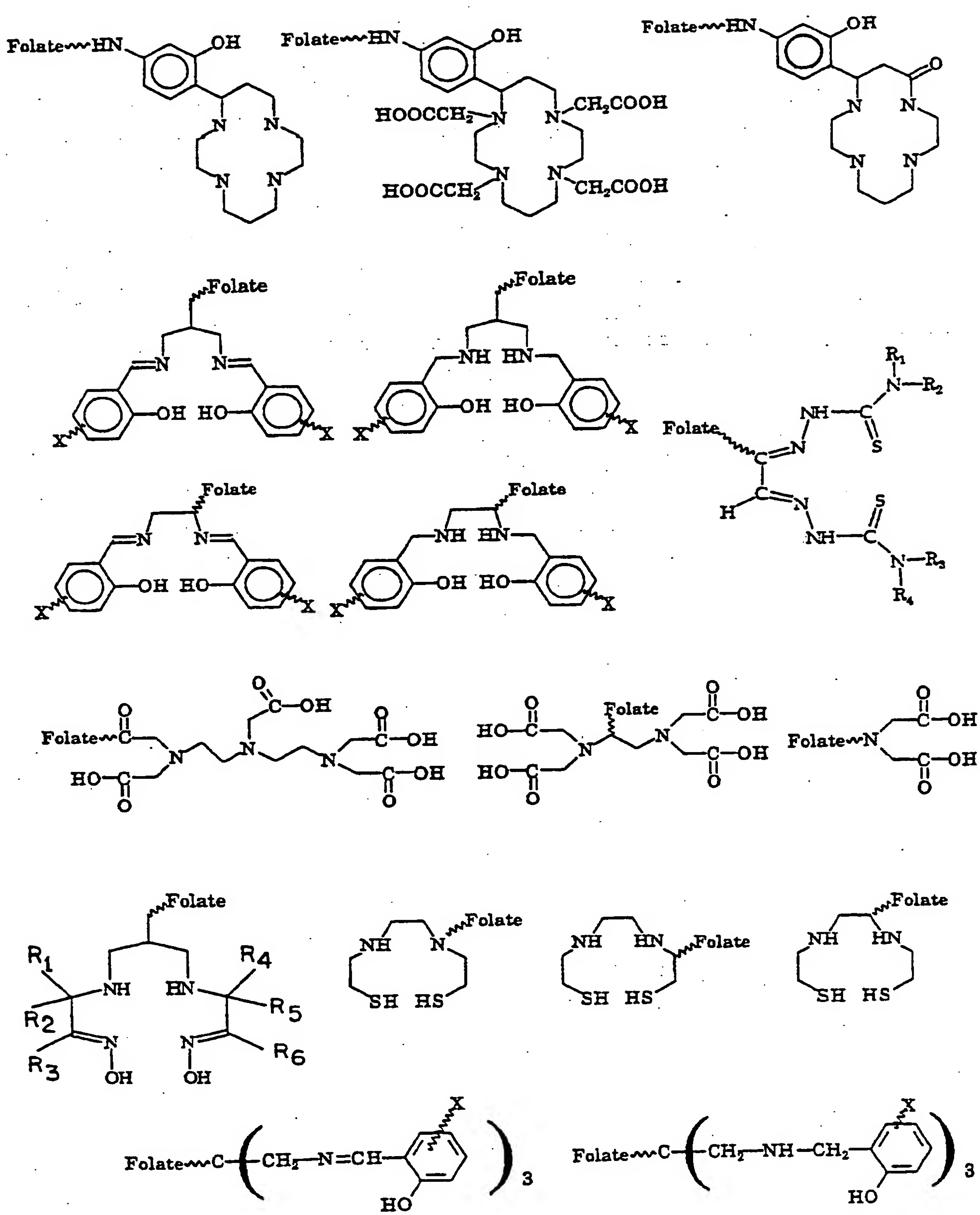
9. The complex of claim 8 wherein the complex comprises a folate molecule and the radionuclide is complexed to the gamma-carboxylate of the folate molecule.

10. The complex of claim 8 wherein the radionuclide is selected from the group consisting of isotopes of gallium, indium, copper, technetium, or rhenium.

11. A complex for imaging tumor cells *in vivo*, said complex comprising a folate molecule or a folate receptor-binding analog of folate; a DTPA molecule covalently linked to said folate molecule or said folate receptor-binding analog of folate; and a radionuclide chelated to said DTPA molecule.

12. The complex of claim 11 wherein the complex comprises a folate molecule and the radionuclide is complexed to the gamma-carboxylate of the folate molecule.

FIG. 1



2/10

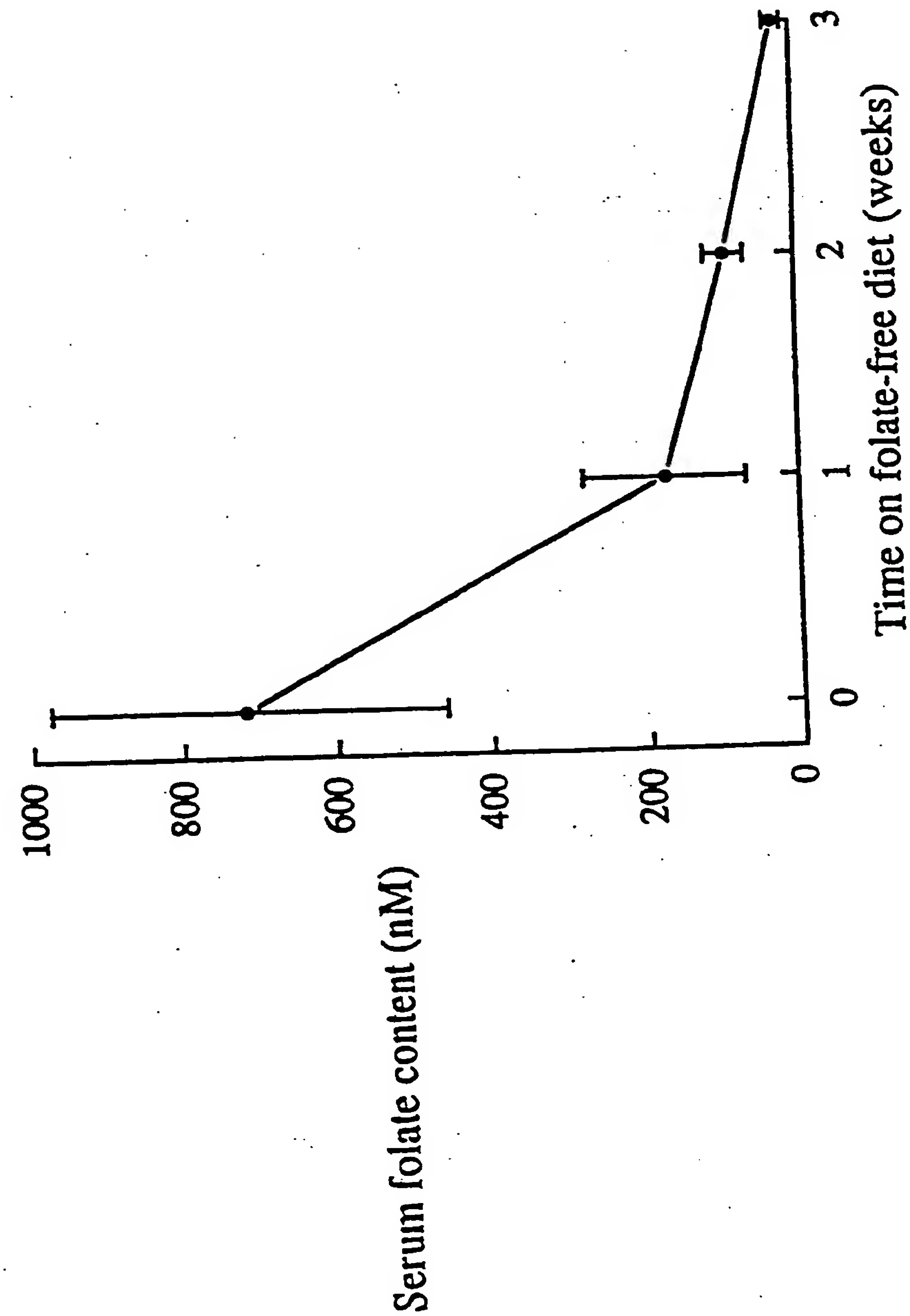
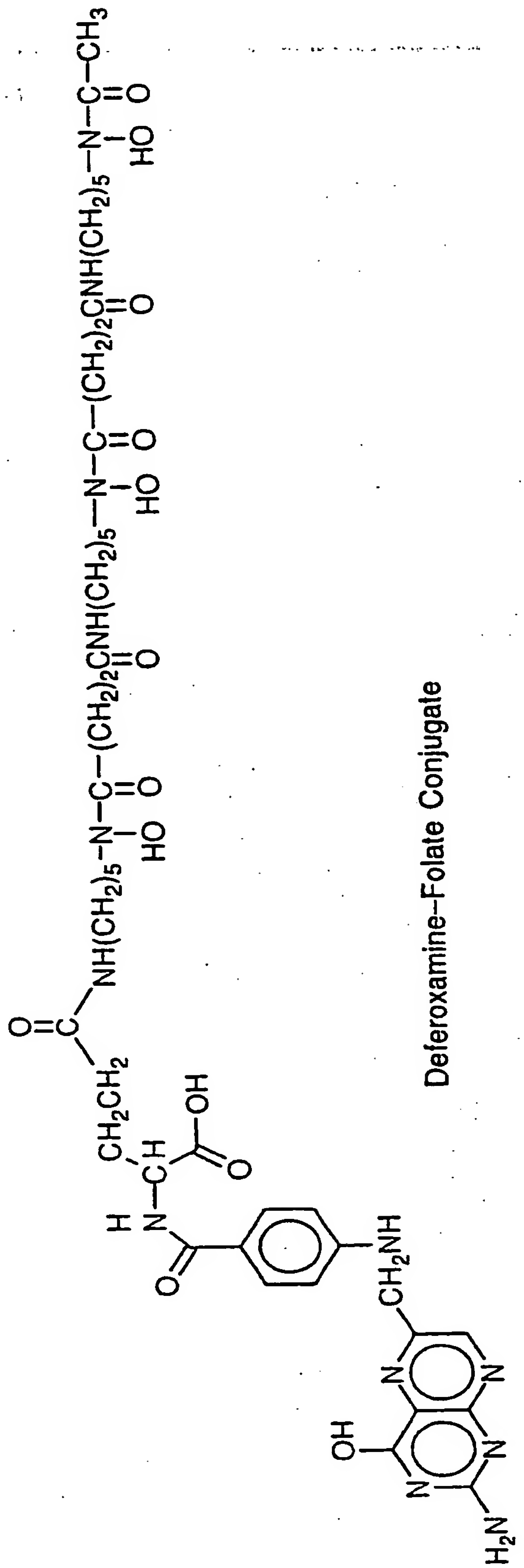

Serum folate content (nM)
1000
800
600
400
200
0
0
1
2
3
Time on folate-free diet (weeks)

FIG. 2



Deferoxamine-Folate Conjugate

FIG. 3

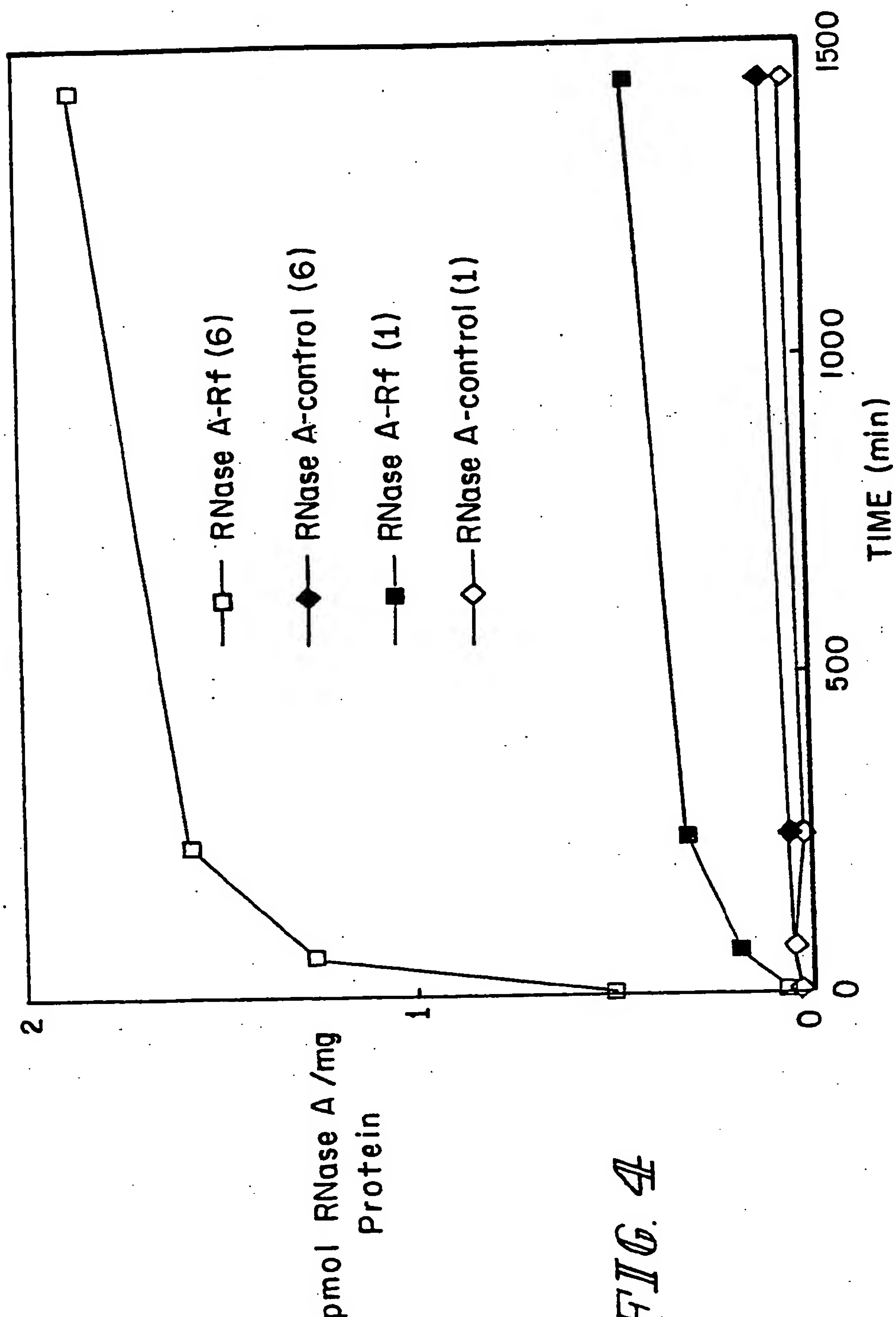
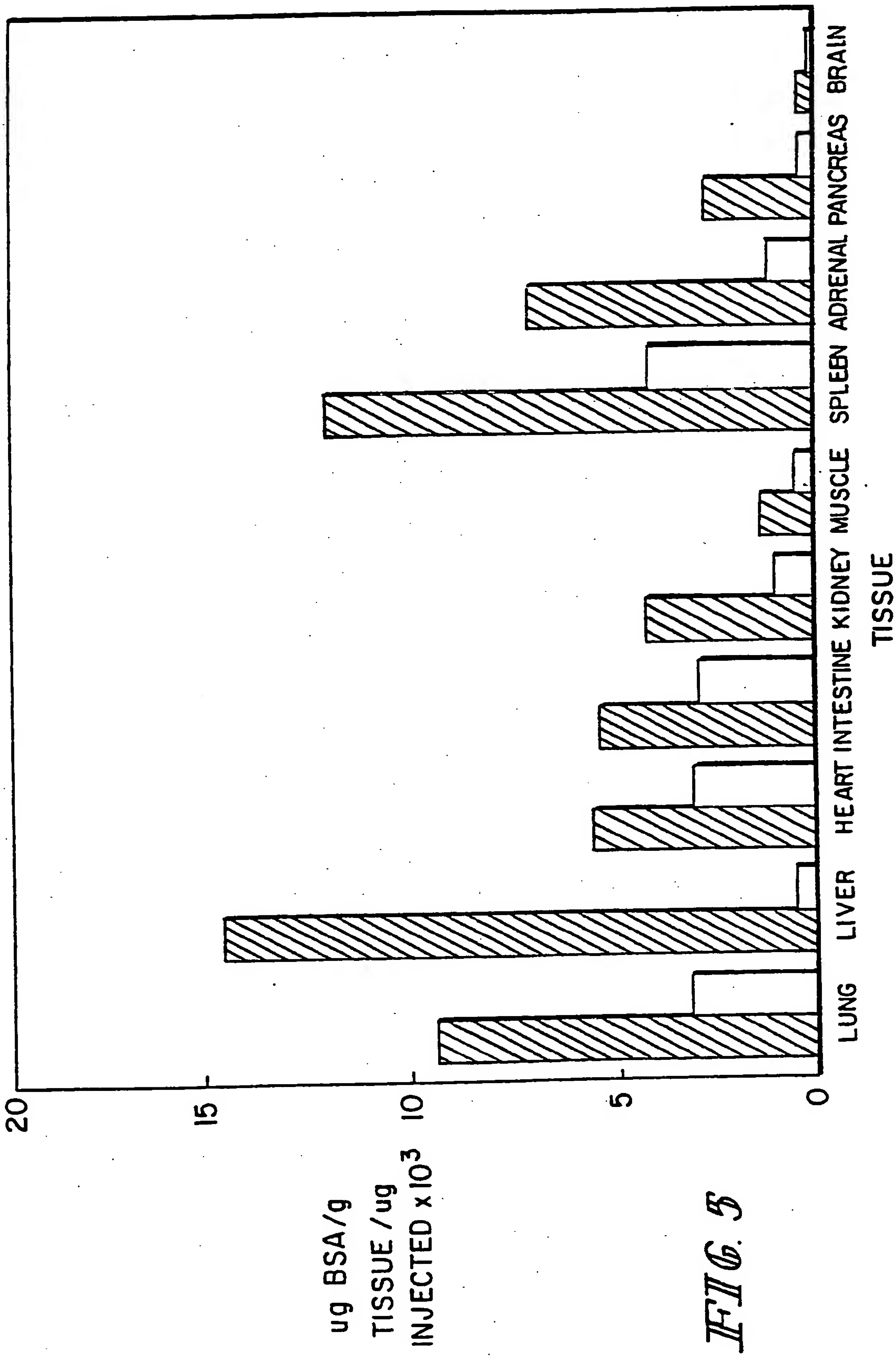

RNase A-Rf (6)
RNase A-control (6)
RNase A-Rf (1)
RNase A-control (1)
pmol RNase A /mg Protein
2
1
0
0
500
1000
1500
TIME (min)

FIG. 4



ug BSA/g TISSUE /ug INJECTED x10^3
20
15
10
5
0
LUNG
LIVER
HEART
INTESTINE
KIDNEY
MUSCLE
SPLEEN
ADRENAL
PANCREAS
BRAIN
TISSUE

FIG. 5

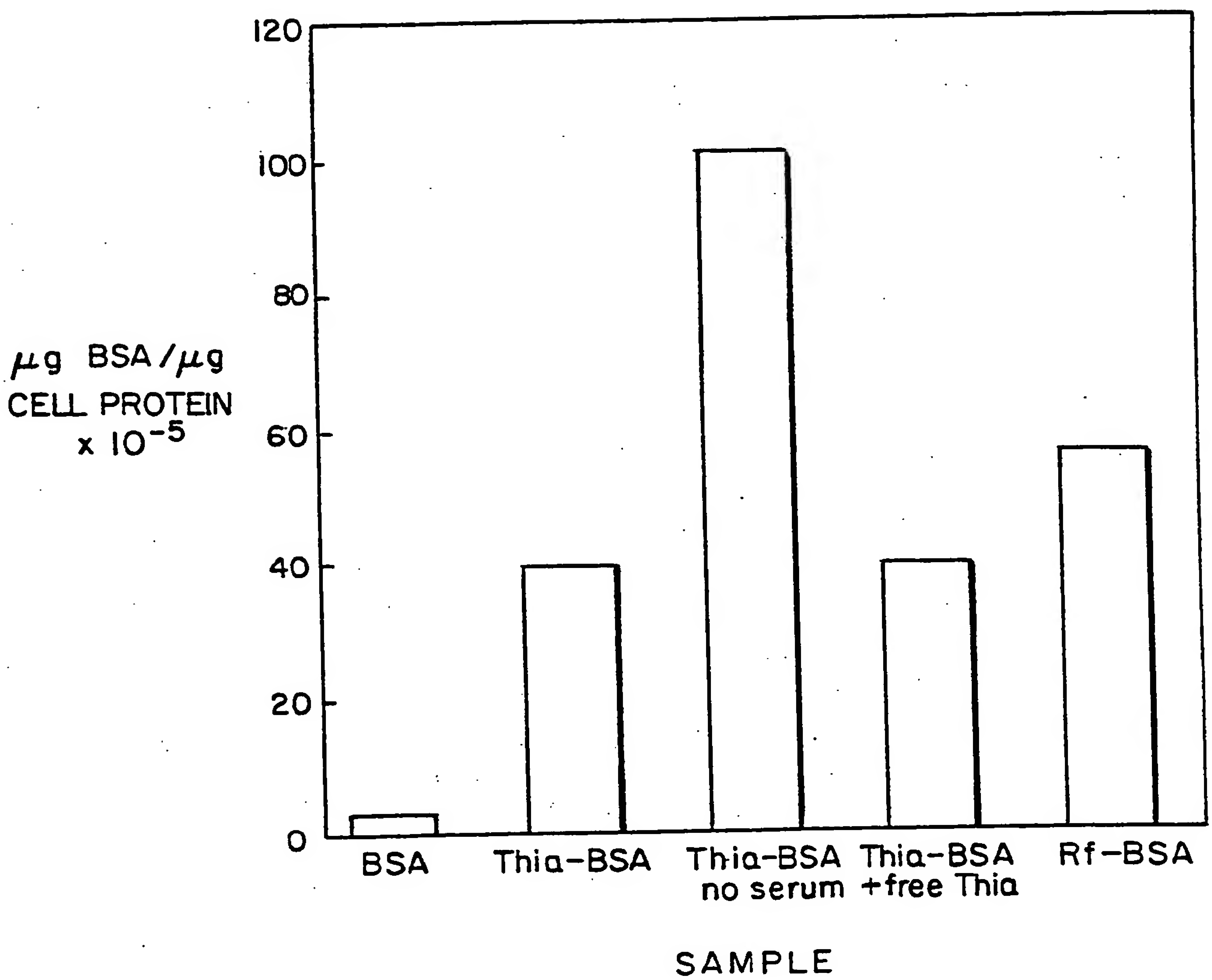

120
100
80
60
40
20
0
μg BSA / μg CELL PROTEIN x 10^{-5}
BSA
Thia-BSA
Thia-BSA no serum
Thia-BSA +free Thia
Rf-BSA
SAMPLE

FIG. 6

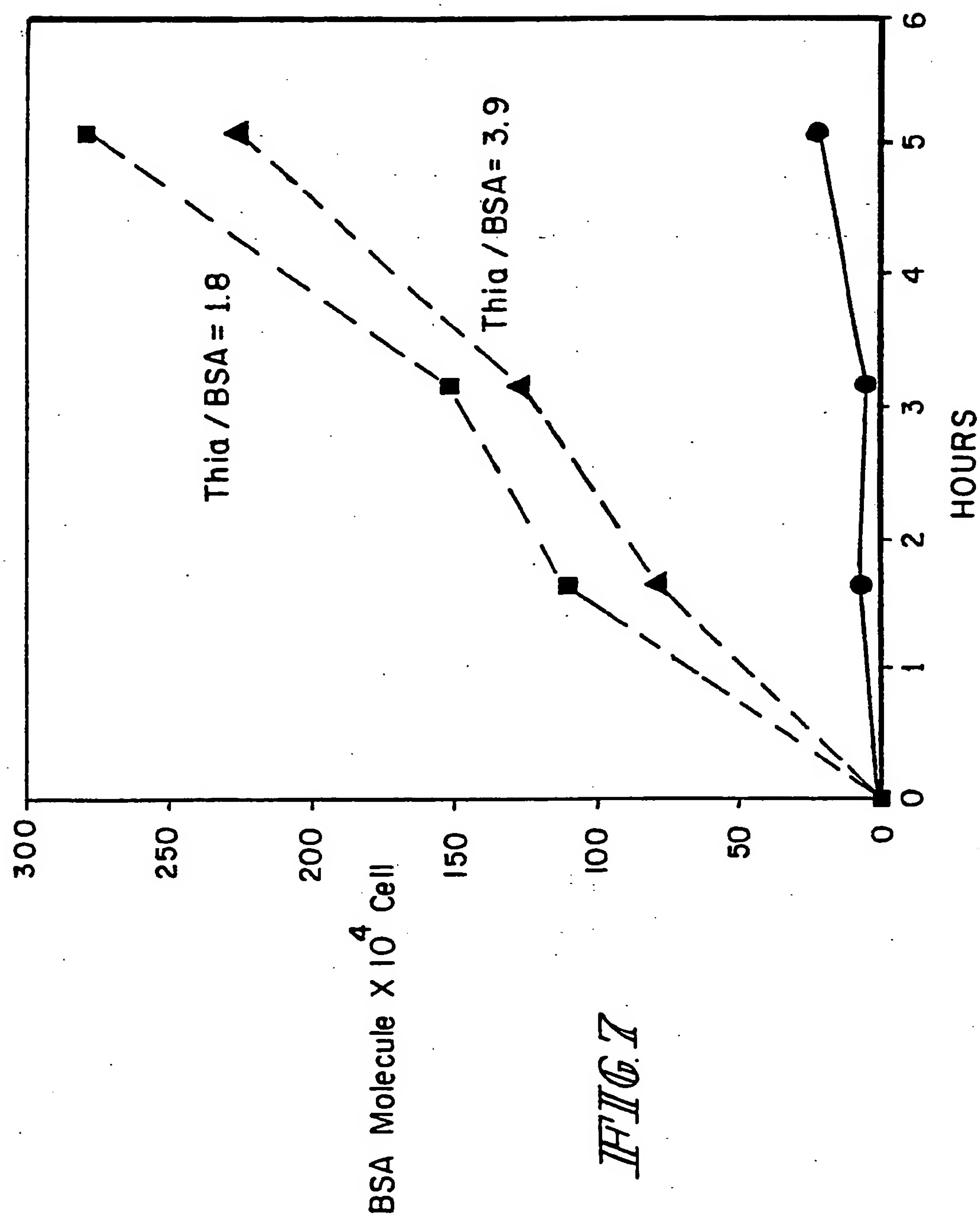

Thia / BSA = 1.8
Thia / BSA = 3.9
BSA Molecule X 10^4 Cell
HOURS
0
1
2
3
4
5
6
300
250
200
150
100
50
0

FIG. 7

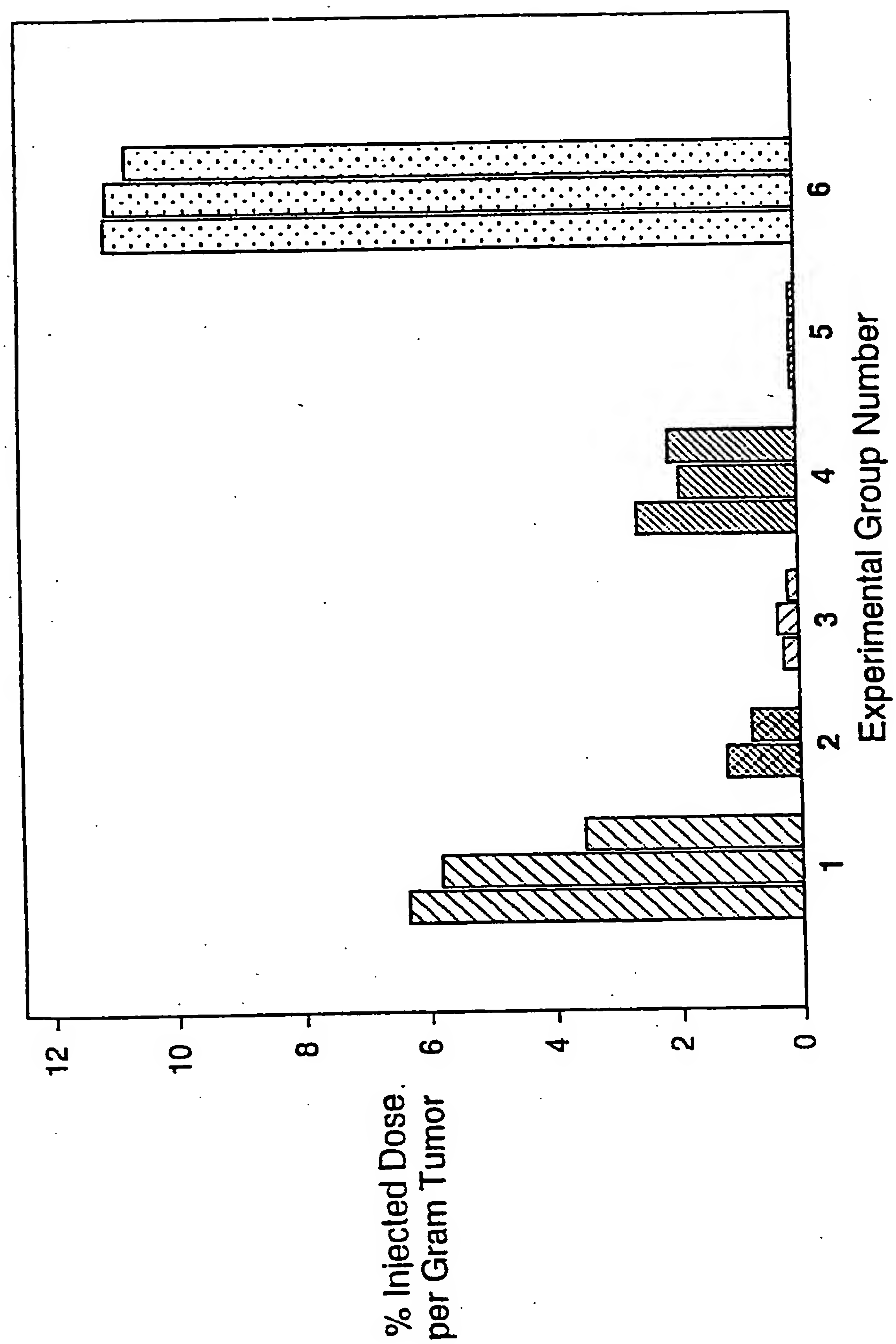

% Injected Dose
per Gram Tumor
0
2
4
6
8
10
12
1
2
3
4
5
6
Experimental Group Number

FIG. 8

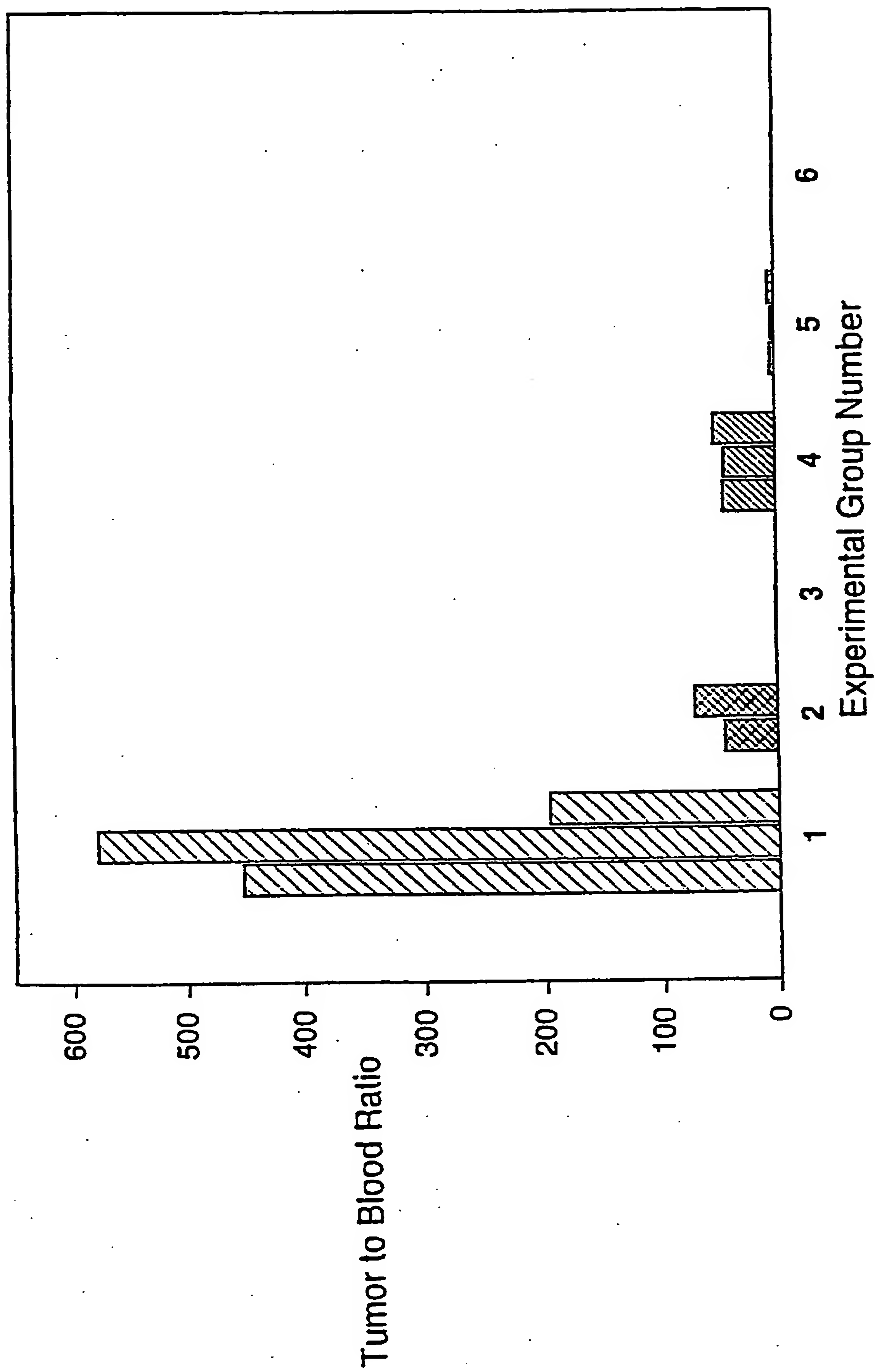

Tumor to Blood Ratio
600
500
400
300
200
100
0
1
2
3
4
5
6
Experimental Group Number

FIG. 9

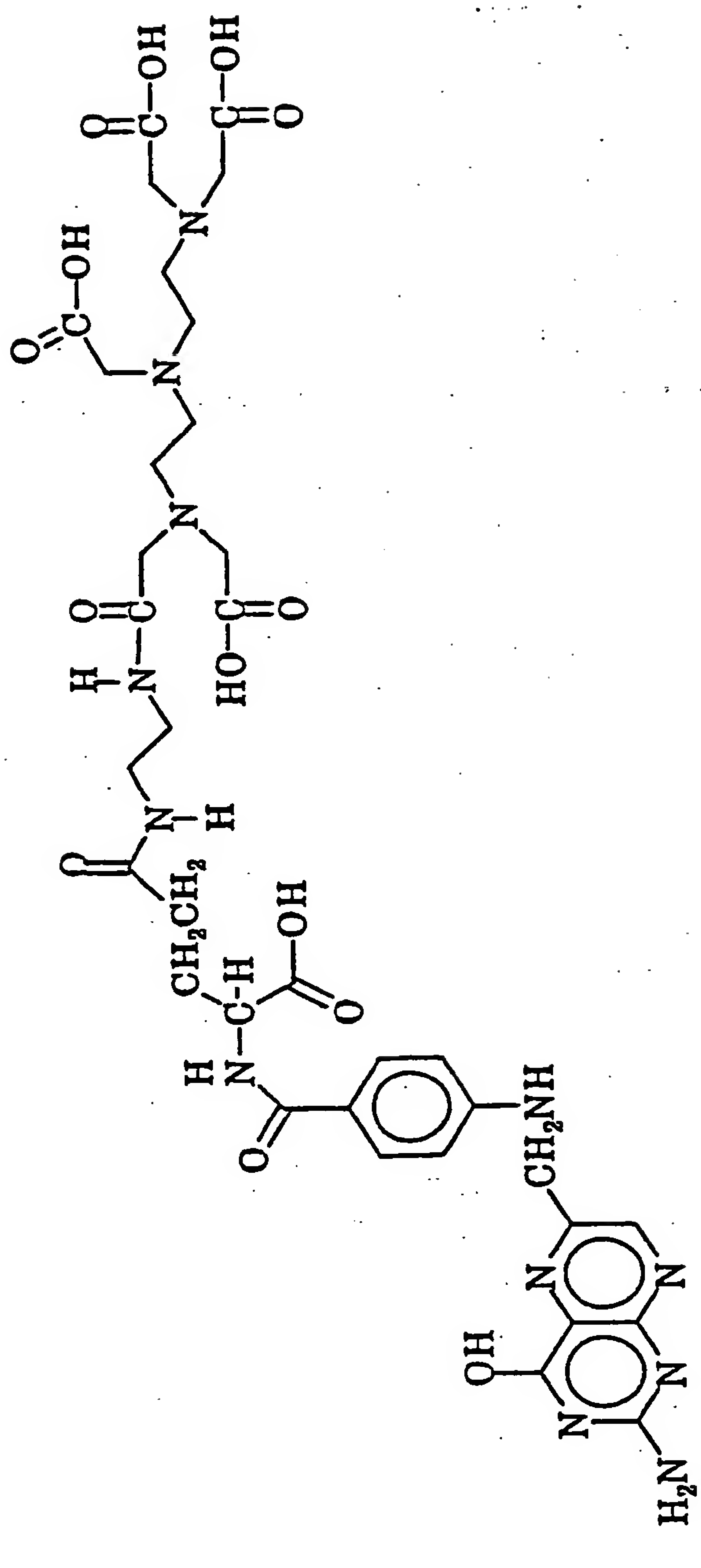


FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/07002

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 51/00, 51/04; C07F 5/00
US CL :424/1.65, 1.41, 1.49, 1.53, 1.69, 9.3; 534/10, 14, 15
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/1.65, 1.41, 1.49, 1.53, 1.69, 9.3; 534/10, 14, 15

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, MEDLINE, BIOSIS
search terms: folate, folic acid, dtpa, deferoxamine, radiodiagnosis, etc.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,094,848 A (BRIXER) 10 March 1992 (10.03.92), see column 2, lines 20+, and column 12, lines 51-65 and column 17, lines 4-15.	1-12
Y	US 5,336,506 A (JOSEPHSON ET AL.) 09 August 1994 (09.08.94), column 1, lines 27-41 and column 8-9.	1-12
Y	US 5,373,093 A (VALLARINO ET AL.) 13 December 1994 (13.12.94), see entire document, especially column 11, lines 50-66.	1-12
Y	US 5,399,338 A (BORN ET AL.) 21 March 1995 (21.03.95), see column 12, especially lines 55-60.	1-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 JULY 1996	15 JUL 1996

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Michael G. Hartley Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)★